FACTORS INFLUENCING THE IMMUNE RESPONSE

by

Robert Neal Pinckard

B.A. Bacteriology, University of Kansas

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To my wife,

for her help,

encouragement and understanding

Preface

The mechanisms involved in the *in vivo* synthesis of the immune globulins has been and still is one of the most perplexing problems to the immunologist. There is now a vast and ever increasing amount of information concerning the synthesis of the peptide chain and the linking together and folding of different peptides chains to form the intact protein molecule. The antibody combining site has been characterized with respect to size, binding affinity and position on the antibody molecule; also certain biological characteristics such as complement fixation and the ability of certain antibodies to fix to tissue have been ascribed to specific portions of the antibody molecule. However, little is yet known about factors which control the specific synthesis of antibody at the cellular level with respect to 1) the recognition and processing of antigen and the proliferation of the sensitized immunocyte, 2) the factors governing the quantity and quality of antibody which is produced following the primary, secondary and tertiary exposure to antigen and 3) factors (either naturally occurring or artificially induced) which tend to completely suppress, partially suppress, or augment antibody production. Operationally it was felt that the second and third areas were most easily approachable experimentally and would yield information which then could be related to the first area. Therefore it was decided to study factors which tended to completely suppress, partially suppress

or augment antibody production with respect to the quantity and quality of the antibody produced.

C O N T E N T S

Page

MATERIALS AND METHODS

RESULTS

"In immunology, we deal with a microcosm that reflects vividly all the essential features of the biological cosmos."

Sir F. M. Burnet

INTRODUCTION

Historical Observations of Contemporary Importance

"Since Jenner made his great discovery of the protective action of vaccinia against small-pox, a century has passed away. During these years that terrible scourge of mankind has been almost completely eradicated from the civilized world. The beneficial consequences of Jenner's discovery are so evident to all who have any wish to properly appreciate them, that one wonders why, during so great a portion of the long period of 100 years, they were allowed to stand alone, without any endeavor being made to induce an artificial immunity in the case of other infectious diseases. This is all the more remarkable because Jenner's discoveries demonstrated in their entirety those essential principles which, in later times, have been established for other infectious diseases." These words were written by Paul Ehrlich in 1900. At the outset of this dissertation one cannot help but look back for 100 years and re-phrase Ehrlich's above statement substituting his name and others from the same period for Jenner's name and "mechanisms of the immune response" for "infectious diseases". It may not be true that the concepts and observations of the early 1900s "were allowed to stand alone"; however, it is of interest that Ehrlich's side chain theory contains many similarities to the clonal selection theories of antibody formation of the last 10 years. Indeed a great part of the research being carried out today has stemmed directly from observations made over 50 years ago. This first section shall therefore be devoted to some of these earlier observations.

Relation of the lymphoid cell family to immunity

Deutsch (1899) was one of the first to demonstrate the relation of lymphoid tissue to specific acquired immunity. He showed that spleen fragments from guinea pigs previously immunized with typhoid bacillus, would transfer immunity to normal guinea pigs 3 - 5 days after transplantation. It was also shown that splenectomy resulted in a decrease of immunity. Twelve years later Luckhardt and Beckt (1911) showed in dogs that the ability to form agglutinating anti-sheep cell antibody could be transferred to unsensitized recipients by the injection of spleen cells from dogs sensitized to sheep erythrocytes. The most dramatic work on passive transfer of immunity via sensitized lymphoid cells was the classic paper of Topley (1930). It was shown that spleen cells from rabbits immunized with Salmonella paratyphii B could confer immunity upon transfer into normal rabbits. The possibility of contaminating antibodies being injected was ruled out by parallel serum transfer studies. Topley noted that the S. typhii had to be injected at least 24 hours before removal and transfer of the spleen cells. The peak antibody response in the recipient rabbits was 6 days after receiving the sensitized spleen cells and disappeared rapidly thereafter. He also observed that after a period of 40 days, the recipient animals did not respond to a greater degree than control animals when given a further injection of antigen. Thus from these studies emanate the characterization of homograft rejection although at that time Topley was not aware of this process.

Transplantation immunity

The genetic aspects of cellular antigens is of paramount importance in hetero or homograft rejection. It was realized around 1900 that erythrocyte antigens were controlled by dominant Mendelian genes. From this data knowledge of the histocompatibility antigens began to develop (Little and Tyzzer, 1916). Shortly thereafter it was found that splenic tissue of inbred Waltzing mice would grow in the F_1 hybrid generation of Waltzing x Albino mice; the reverse situation resulted in rejection (little and Johnson, 1922). The successful transplantation of tissue between monozygote twins was accomplished by Bauer in 1927 and Padgett in 1932 (see Hašek, Lengerová and Hraba, 1961), also stressing the genetic influence upon graft survival. The first observations of the graft versus host reaction were described by Murphy (1916) and Danchakoff (1916). It was observed that transfer of spleen, bone marrow, liver or kidney from adult chickens into chicken eggs would cause a characteristic splenomegaly and leucocytosis (Murphy, 1916). The immunologic causality of graft rejection, although proposed by Schoene in 1912, was not put on a firm basis until Gibson and Medawar (1943) demonstrated accelerated second set rejection of skin transplants. Of major importance today in the field of transplantation immunity is the study of anti-lymphocyte serum which may prolong skin and kidney grafts. The destruction of white cells by anti-lymphocyte serum, which may be the mechanism of prolonged graft survival, was demonstrated in 1899 by Metchnikoff, followed shortly thereafter by

similar observations by Besredka in 1900 and by Christian and Leen in 1905 (see Gray, Monaco, Wood, and Russel, 1966).

Characterization of the immunoglobulins

The characterization of antibody with respect to immunoglobulin class and dynamics of synthesis is a relatively more recent development. The explanation for this was the lack of physical and chemical methods to separate the various components of serum. In 1901 Hiss and Atkinsin noted that the globulin fraction of diphtheria anti-toxin sera possessed more protein when compared to normal sera. Glenny and Sudmersen (1921) reported differences between the kinetics of the primary and secondary immune responses to diphtheria toxin. Earlier Glenny (1913) had reported two forms of diphtheria anti-toxin of different avidity. In the mid 1930s it was established that antibody globulin could vary with respect to molecular weight. Goodner, Horsfall and Bauer (1936) reported that antibodies to Type 1 pneumococcus possessed varying molecular weights as demonstrated by ultrafiltration. Heidelberger, Pedersen and Tiselius (1936) and Heidelberger and Pedersen (1937) noted differences in the molecular weights of horse and rabbit anti-pneumococcal sera by ultracentrifugation. In 1937 Tiselius reported the technique of moving boundary electrophoresis thus characterizing the α, β, and γ globulins. Shortly thereafter Tiselius and Kabat (1939) studied antibodies in the horse, rabbit, cow and monkey which revealed differences in electrophoretic mobility. In the horse two antibody

components were separated; the initial component to be synthesized (T-globulin) was of high molecular weight and migrated between the β and γ globulins but subsequently a different antibody developed with a slower mobility. Further elucidation of the temporal synthesis of antibody had to wait until the late 1950s for techniques such as immunoelectrophoresis, sucrose density gradient ultracentrifugation and column chromatography to be developed before further progress was to be made.

Avidity of antibody

The avidity of antibody, i.e., the relative ability of antibody to form stable complexes with antigen, was first noted in 1900 by Roux and in 1904 by Cruveilhier. In 1903 Kraus introduced the term "avidity". The preceding work concerned the diphtheria toxin-antitoxin system. Glenny (1913) noted two forms of diphtheria anti-toxin with differing avidities for the lethal constituent of diphtheria toxin. In 1932 Glenny and Barr devised a dilution test which enabled the avidity of diphtheria anti-toxin to be accurately delineated. More recently Barr (1951) has reviewed various factors which tend to influence avidity, such as the method of primary and secondary immunization procedures. The study of avidity has become the object of major research efforts during the past ten years; further discussion of this phenomena will be given later.

Augmentation of antibody production

It soon became apparent to the early immunologist that the quantity of antibody produced could be increased by various procedures. Glenny and Sudmersen (1921) found that complexes of diphtheria toxin-antoxin could augment immunity. They also observed that the secondary response was of greater magnitude only when the secondary stimulus was given long after the primary injection. Glenny, Pope, Waddington and Wallace (1926) later demonstrated that diphtheria toxin was more immunogenic when absorbed onto alum. By injecting antigen into animals suffering from tuberculosis Dienes (1927) obtained enhanced antibody production to ovalbumin; Lewis and Loomis (1924) noted elevated agglutinin formation when sheep erythrocytes were injected into tuberculous animals. Clark, Zellmer and Stone (1922) demonstrated enhanced antibody production to S. typhosa by simultaneously injecting gram positive cocci; they related their findings to the apparent increased resistance to infection of humans living in urban as opposed to rural areas. Previous injection of coli, paratyphii B or pyocyaneus bacillus increased antibody production to Gaertner's bacillus (S. enteriditis) (Khanolkar, 1924); sodium nucleinate and sodium cinnamate likewise enhanced antibody production to Gaertner's bacillus. The adjuvant effects of certain materials will be discussed at greater length later.

Suppression of antibody production

The suppression of antibody formation is a complex and

diversified area of study which today still remains obscure. Smith (1909) first noted that passive antibody suppresses antibody formation; later Glenny and Sudmersen (1921) using the same model found that antibody to diphtheria toxin may suppress the primary response to diphtheria toxin. Wells and Osborne (1911), by feeding animals with zein, ovalbumin and bovine serum reduced the susceptibility to anaphylaxis. Benjamin and Sluka in 1908 found that X-irradiation would suppress precipitin formation if the radiation was given prior to the antigenic stimulus (see White, 1963). Sulzberger (1929) prevented sensitization to arsphenamine by intravenous injections of the same compound prior to the sensitizing injection. Competition of antigens has long been realized, first being noted in 1902 by Michaelis (see Adler, 1959). Perlzweig and Steffen (1923) first demonstrated polysaccharide paralysis; later Felton and Ottinger (1942) further characterized this suppression. Unresponsiveness to protein antigens was clearly demonstrated by Glenny and Hopkins (1924) who found that daily injections of horse diphtheria anti-toxin led to a decrease in elimination from the body when compared to animals receiving weekly injections. In other reports by Glenny and co-workers suppression of antibody production by improper immunization procedures was noted (see Barr, 1951). More detailed accounts of unresponsiveness will be cited later.

This brief resume has been presented in order to cite only some of the earlier observations which have served as the foundation of contemporary research. It is incomplete in many

respects but a rigorous description of all of the historical observations is beyond the scope of this section. It is fascinating and indeed humbling to realize how much of today's research is based directly on observations of 30 - 100 years ago. Perhaps these original observations will be clearly understood in the not too distant future; however, this will be possible only by employing more recent techniques which have enabled the immunologist to work at the molecular and chemical level.

Factors Enhancing Antibody Formation

Substances or treatments which tend to augment antibody formation will be described as possessing "adjuvant" qualities. The augmentation of antibody production may be reflected by the increased quantity or quality and/or accelerated production of antibodies. The adjuvant properties of many materials have been known since early in the history of immunology; some earlier work has been previously described. A comprehensive review of the spectrum of substances defined as adjuvants and their possible mode of action is beyond the scope of this section; more detailed accounts may be found elsewhere (White, 1963; Munoz, 1964; Herbert, 1964).

Studies on the adjuvant properties of bacteria and bacterial products have formed the backbone of knowledge concerning enhancement of antibody formation. The gram negative and acid-fast bacteria have been extensively employed

as adjuvants but their mode of action is still a matter of speculation. The differences encountered when studying the mode of action of various adjuvants arise in part from variables such as type of antigen used, number and route of antigen injections, species, strain and age variations, nutritional differences and a wide variety of environmental factors. In general, adjuvant activity may operate by 1) altering the physiology of the animal and 2) prolonging the antigenic stimulus. The second mechanism may be decisive in bringing about greater quantities of antibody over an extended period of time but does not account for the immediate adjuvant effects of many substances.

The toxic and/or lymphoreticular stimulatory effects of many substances have been strongly implicated as the mechanism of adjuvant action. Johnson, Gaines, and Landy (1956) reported that the adjuvant effect of the lipopolysaccharide from S. typhosa was correlated to the "stress symptoms" it produced in rabbits. The mouse and guinea pig are fairly resistant to this endotoxin and little adjuvant activity was noted in these two animals; also rabbits made tolerant to the endotoxin showed no enhanced antibody production. Kind and Roffler (1961) and Farthing and Holt (1962) also demonstrated minimal adjuvant effects in animals which had received endotoxin prior to challenging with endotoxin and antigen. White, Coons and Conolly (1955, 1955a) studied the adjuvant properties of alum and Freund's adjuvants and indicated that, besides a prolonged release of antigen, widespread proliferation of

plasma cell elements in the spleen, liver and lymph nodes, and local macrophage proliferation played an important role in enhancing antibody production. Kind and Johnson (1959) postulated a critical period of antibody production that endotoxin affected, which possibly involved the primitive reticular cell. This was suggested in view of the finding that endotoxin could reverse the suppressive effects of X-irradiation (Kind and Johnson, 1959) and cortisone administration (Ward and Johnson, 1959) and that endotoxin was only active if given shortly before or after antigen. Reticular cell proliferation and function has recently been shown to be dependent upon environmental stimuli such as bacteria (Bauer, Paronetto, Burns and Einheber, 1966). Fisher (1966) has recently reported enhancement of haemolysin formation by using india ink, saccharated iron oxide, thorotrast and polystyrene latex as adjuvants; all four of these materials caused splenomegaly and aggravated the reticuloendothelial system thus causing its stimulation.

Endotoxin has also been suggested as a cause of other effects which may augment antibody production. Condie, Zak and Good (1955) suggested that endotoxin, besides activating reticular cells, may alter vascular permeability. One of the most lucid observations of increased membrane permeability due to endotoxin was the work of Weissman and Thomas (1962). They demonstrated that endotoxin affects the liver lysosomal membranes causing a release of the two hydrolytic enzymes, β - glucuronidase and cathepsin. Isolated lysosomes from rabbits

tolerant to the endotoxin or given hydrocortisone did not release the two enzymes.

Paradoxically many treatments and substances which suppress antibody formation may, in certain instances, enhance antibody production. Taliaferro and Taliaferro (1954), Graham, Graham, Neri and Wright (1956) and Dixon and McConahey (1963) demonstrated the adjuvant effects of X-irradiation; enhancement was found to vary with antigen dose and was greatest when 400 - 600 r X-irradiation was given 2 - 3 hours after antigenic stimulation (Dixon and McConahey, 1963). Cortisone and prednisolone treatment prior to antigenic stimulation enhanced antibody formation if the steroid treatment was stopped a few days before immunization (Winter, Diefenbach, Strauch and Stender, 1962). Colchicine augmented haemolysin production in X-irradiated and normal rabbits (Taliaferro and Jaraslow, 1960). Claman and Bronsky (1965) found that in mice actinomycin D enhanced antibody production to BGG. Compounds such as 5-fluoro-2-deoxyuridine, uracil mustard and cyclophosphamide have also been shown to stimulate antibody production (Merrit and Johnson, 1963; Buskirk, Crim, Petering, Merrit and Johnson, 1965). Recently Chanmougan and Schwartz (1966) demonstrated enhanced antibody production when antigen was given five days after 6-mercaptopurine.

There have been a number of reports of DNA and RNA or their analogues enhancing antibody production or restoring the immune capacity of X-irradiated animals. Taliaferro and Jaraslow (1960) restored haemolysin formation in X-irradiated

rabbits by injecting DNA and RNA digests with antigen 24 hours after X-irradiation. Johnson, Hoekstra and Merrit (1965) reported enhancement of the primary response to BGG by heterologous DNA, RNA, histones from thymus and spleen, DNase treated DNA and combinations of various nucleic acid analogues. Braun, Nakano, Hechtel, Plescia and Diskov (1965) and Braun (1965) reported that oligonucleotides from calf thymus increased the number of plaque forming cells to sheep erythrocytes.

The myriad of substances which may augment antibody production is startling; indeed it appears that practically anything may possess the ability to enhance antibody production. It is worth pointing out that many adjuvant-like materials generally stimulate only the primary response; therefore one could assume that they enhance the early cellular mechanisms involved in the recognition and/or sensitization phases of antibody production rather than the proliferative phase. The point will be made later that adjuvants may act not by augmenting the antibody forming mechanisms, but may decrease the tolerance inducing mechanisms by accelerating the early mechanisms of antibody formation. Many adjuvants do in fact appear to stimulate cellular physiology in general or result in a release of analogues responsible for protein synthesis and cellular proliferation. The problems concerning the mode of action of adjuvants were summarized by Munoz (1964) who said, "It is clear that the exact modes of action of bacterial adjuvants, or adjuvants in general, are not definitely known. The phenomenon is complex and cannot be expected to have a

simple answer. There are many factors which affect the antibody response and many ways in which the antibody response can be modified. It can be safely said that adjuvants of different kinds act differently and that any one adjuvant may have more than one mechanism by which it increases antibody formation."

Factors Influencing the Quality of Antibody

"Avidity" and "relative binding affinity" will be used to describe the change in the quality of antibody. Although both of these terms are inter-related, subtle differences between the two exist. Relative binding affinity is defined as the magnitude of the average intrinsic association constant, K_o, of an antibody combining site and its specific antigen combining site (see Pinckard and Weir, 1967). Therefore the relative binding affinity is solely dependent upon the primary interaction of antibody and antigen. Avidity on the other hand describes the relative stability of antigen-antibody complexes; however, this is dependent upon both the primary and secondary reactions of antigen and antibody (see section of "Detection and characteristics of humoral antibody production" for the descriptions of primary and secondary reactions of antibody and antigen). The differences between avidity and relative binding affinity may be more easily viewed thermodynamically. Relative binding affinity is directly related to the standard free energy change of the

primary intrinsic interaction between antibody and antigen. Avidity, however, is related to the free energy change of complex formation and other factors occurring during secondary reactions; therefore avidity may encompass a greater thermodynamic stabilization, such as more involved entropy changes due to the transformation of soluble antigen-antibody complexes from a liquid state into a solid state precipitate, i.e., a decrease in entropy. Therefore although avidity and relative binding affinity are often used interchangeably, it must be realized that differences do exist.

The quality of antibody termed avidity was first realized through the studies of diphtheria anti-toxin as mentioned earlier. It was originally noted that antisera varied in their ability to neutralize or precipitate the toxin. Later it was found that avidity could be quantitatively assessed by diluting the original toxin-antitoxin mixture which would reverse the interaction to varying degrees depending upon the avidity of the antiserum. Barr (1951) and Jerne (1951) further characterized the avidity of the diphtheria toxin-antitoxin reaction; Raynaud (1959) has recently reviewed the heterogeneity of this system.

Studies on the production of erythrocyte agglutinins and haemolysins have also revealed differences in avidity. It had been long observed that lysis of erythrocytes resulted in an irreversible inactivation of alexin or complement, but antibody could dissociate and cause further lysis with fresh complement. With this knowledge Taliaferro, Taliaferro and

Pizzi (1959) employed chromium^{51}-labelled erythrocytes to measure the intercellular transfer of haemolysins. They found that as the time after the initial immunization increased, the avidity of the haemolysins increased as reflected by lack of transfer of haemolysin from erythrocyte to erythrocyte; it was postulated that low-avid antibody may thus be more biologically active than high-avid antibody. Goodman and Masaitis (1960) using a similar test found that non-avid haemolysins were γ_2 globulins whereas high-avid antibodies were γ_1 globulins. More recently Greenbury, Moore and Nunn (1965) demonstrated differences in avidity between rabbit 7S haemolysins and its subunits; it was found that the Fab fragment or the $F(ab)_2$ recombinant formed by antibody Fab and non-antibody Fab possessed lower avidity when compared to the 7S, $F(ab)_2$ fragment or $F(ab)_2$ antibody recombinant. This last paper thus illustrates that avidity may be dependent upon the number of antibody combining sites per antibody molecule; the relative binding affinity, however, is intrinsic for the antibody combining site.

Virus neutralization tests have been used to assess the avidity of antibody by reactivation procedures. Andrews and Elford (1933) first noted that coliphage could be reactivated by dilution of the coliphage-anticoliphage mixture. Jerne and Avegno (1956) renewed this earlier work studying the inactivation of T_4 phage by the decision tube assay method. It was found that in early antisera a large percentage of phage could be reactivated by dilution whereas only a small

percentage could be reactivated when late sera were employed. Lowering the incubation temperature or ionic strength of the reaction media decreased the reactivation rate; both of these procedures favour thermodynamic stability of the antigen-antibody complex. Svehag (1965) characterized the increase in avidity (post-immunization) of antibody to polio virus. Avidity was assessed by the extent to which the virus would dissociate from antibody in the presence of acid, pH 2.5 - 6.0. It was found that early 19S antibody (γ_1-globulin) was less avid than late 19S (γ_1-globulin) antibody; the 7S antibody appearing later in immunization had high avidity. Recently Finkelstein and Uhr (1966) reported increase in avidity post-immunization in both 19S and 7S antibodies to ϕX174 coliphage; it appeared that at the same stage in immunization that 19S antibody was more avid than 7S antibody. This last point could be ascribed to the greater number of combining sites on the 19S antibody molecule.

Antibodies to soluble heterologous serum proteins also vary with respect to avidity and relative binding affinity. Talmage and Maurer (1953) noted that as the time increased after initial sensitization with bovine gamma globulin, bovine serum albumin and egg albumin, the P^{40}/P^{80} ratio also increased; it was concluded that this was due to the increase in avidity. Talmage (1957) later studied the 50% dissociation time (T/2) of BSA-anti-BSA precipitates; early antibody possessed a T/2 value of 3 hours compared to 72 hours for secondary antisera. The breakthrough in the study of the relative binding affinity

of anti-BSA antibody came from the classic work of Farr (1958). By employing the ammonium sulphate method for determining the antigen-binding capacity, Farr demonstrated the increase in relative binding affinity of anti-BSA antibodies after primary and secondary stimuli and related this to precipitability as measured by the P^{80} test. Talmage (1960) confirmed and extended the previous work of Farr. Branster and Cinader (1961) reported the increased inhibitory action of anti-ribonuclease sera in the later stages of immunization. Grey (1964), employing the Farr technique, demonstrated an increase in the relative binding affinity in rabbits receiving varying amounts of BSA injected by different routes. He also demonstrated that although 19S antibody possesses a smaller association rate than 7S, the dissociation rates of the two antibody molecules were similar.

The most direct approach for studying changes in the relative binding affinity of antibody is to employ haptens. Indeed one may actually characterize the value of K_o by means of equilibrium dialysis or fluorescent quenching. Hooker and Boyd (1941) noted a widened range of reactivity of antibody after prolonged immunization; the increased cross reaction, as demonstrated by inhibition of precipitation, could have only resulted from an increase in the K_o value of the antibody combining site. Differences in K_o values up to a thousand fold were found for the same dinitro-phenyl group (DNP) by Cartsen and Eisen (1955) and Velick, Parker and Eisen (1960). Karush (1962) and Pinckard and Weir (1967) pointed out that

this was likely to be due to the differences in immunization procedures and the hapten/protein ratios of the immunizing antigens. Eisen and Siskind (1964) demonstrated a thousand fold increase in the K_o values from 2 - 6 weeks after the initial immunization with DNP-BGG; if large amounts of antigen were injected no increase was observed. Siskind and Eisen (1965) repeated the preceding work and demonstrated that passive cutaneous anaphylaxis was directly dependent upon the magnitude of the K_o value. Klinman, Rockey, Frauenberger and Karush (1966) have recently demonstrated differences in K_o values between horse IgA and IgG antibody 6 weeks after immunization; 6 months later the K_o value of IgA antibody was the same whereas the IgG antibody value had risen from 5×10^5 litres/mole to greater than 10^7 litres/mole thus equalling the IgA antibody value. Steiner and Eisen (1966) recently reported increase in K_o values using *in vitro* synthesis of antibodies which incorporated radioactive amino acids; they substantiated previous findings and also indicated that the increase of relative binding affinity was not a result of high affinity antibody being selectively removed from the circulation but was due to the sequential synthesis of antibody by lymphoid cells.

Factors Influencing the Induction, Maintenance, and Abrogation of Acquired Immunological Tolerance

Specific suppression of the immune response, although

recognized for over 60 years, did not receive much attention during the first 50 years of this century. The work already mentioned of Wells and Osborne (1911), Glenny and Hopkins (1924), Sulberger (1929), Perlzweig and Steffen (1923) and Felton and Ottinger (1942) laid the foundation for contemporary research on immunological tolerance. Burnet and Fenner (1949) summarized earlier immunological observations and postulated the "self-marker" concept of antigen recognition; they hypothesized that exposure to antigen during embryonic life would lead to unresponsiveness to the same antigen in adult life. This reasoning was primarily the result of work by Owen (1945) who demonstrated erythrocyte chimerism between bovine twins; this occurred from vascular anastomoses of the placentas resulting in the exchange of haematopoietic cells which then were accepted as self.

The terms "tolerance", "paralysis" and "unresponsiveness" have been used to describe the state of specific, acquired immunological negativity to a certain antigen. Tolerance was first used in the context of acceptance of tissue of genetic disparity from the host. Paralysis was coined from reports concerning polysaccharide antigens and unresponsiveness to chemical (hapten) or protein antigens. Many workers today use all three terms interchangeably and feel that all three mechanisms of immunological negativity are similar if not identical. A more conservative view will be taken in the present report and more concise definitions will be employed. Paralysis will not be used as it is felt that this terminology

is vague and does not provide an operational definition of immunological negativity. Tolerance and unresponsiveness will be used synonymously; both terminologies are defined as the specific, acquired immunological negativity which is induced by antigen in the neonatal or adult animal. Moreover, many research workers have used tolerance or unresponsiveness to describe a diminished state of immunological reactivity; in the present review this state will be referred to as partial tolerance. The preceding review, as an introduction to the present studies, will describe only those reports concerning immunological tolerance to soluble, protein antigens; no attempt will be made to compare in detail unresponsiveness to soluble, protein antigens with polysaccharide paralysis or tolerance to tissue antigens because the author is not yet convinced that all three states of immunological negativity reflect the same mechanism.

Induction of unresponsiveness in neonatal animals

The induction of unresponsiveness to a protein antigen was first described by Hanan and Oyama (1954). They demonstrated that rabbits receiving intraperitoneal injections of alum precipitated BSA during the first four weeks of life and intravenous injections thereafter did not produce antibodies to BSA in adult life as demonstrated by passive anaphylaxis and arthus reactions. Dixon and Maurer (1955) induced unresponsiveness to BSA and human plasma by subcutaneous injections of the respective antigens starting at birth and

continuing for 3 - 4 months; the tolerance persisted for 10 - 11 months after the cessation of antigen infusion. The unresponsive state was specific for the injected antigen and was not transferred to first generation offspring. At the same time Cinader and Dubert (1955,1956) induced tolerance to HSA in neonatal rabbits. The tolerance was specific in that adult animals, which were unresponsive to HSA, elicited a normal antibody response to tobacco mosaic virus. Injection of tolerant rabbits with sulphanil-HSA led to the formation of antibodies only to the hapten residue. In contrast Boyden and Sorkin (1962) later demonstrated that rabbits rendered tolerant to HSA at birth would not produce either hapten specific or protein specific antibodies when challenged in adult life with sulphanil-HSA; however, HSA tolerant rabbits did produce hapten specific antibodies when challenged with sulphanil-BSA or sulphanil-rabbit albumin. Attempts to induce tolerance to sulphanilic acid in neonatal rabbits by the injection of 10 mg sodium sulphanilate failed.

Smith and Bridges (1956,1958) fully characterized the tolerance-induction period in the neonatal rabbit with respect to dosage. It was found that a single intraperitoneal injection of between 10 - 100 mg of BSA, HGG, EA or human macroglobulin produced an unresponsive state to the respective antigen lasting 90 - 120 days; feeding neonatal rabbits with 100 mg BSA also induced a tolerant state of similar duration. Passively transferred lymphoid cells from immunized rabbits to tolerant rabbits or transfer of lymphoid cells from tolerant

rabbits to normal rabbits did not break tolerance. Neonatal rabbits had to be less than 14 days of age in order to regularly induce a firm and lasting tolerance to protein antigens (Eitzman and Smith, 1959; Smith, 1961). Humphrey (1964) rendered rabbits unresponsive to HSA, BSA and HGG by neonatal injection. He found that rabbits continued to be tolerant to the antigen long after the original antigen, injected at birth, had theoretically disappeared from the circulation. Passively administered antibody failed to abrogate the unresponsive state. Rabbits which finally produced detectable antibody elicited a very weak response and upon further injection of antigen, in many cases, returned to an unresponsive state.

Terres and Hughes (1959) induced tolerance in neonatal mice by injecting 50 mg BSA within the first three days of life; the tolerance was still present after 6 weeks but not after 12 weeks and it was found that 12.5 mg BSA was not able to induce unresponsiveness to BSA. Eitzman and Smith repeated and extended the above work using a single injection of 18 mg or 36 mg BSA at birth and found the tolerance to persist for 7 weeks (see Smith, 1961). Dresser (1961) produced tolerance in neonatal mice lasting 4 months and partial tolerance at 6 months after injection of 10 mg BGG within the first 12 hours of life. The tolerance could be maintained in the adult mouse by further injection of soluble BGG.

Recently Nossal and co-workers have initiated a number of

studies concerning the induction of tolerance to the soluble flagellin of Salmonella adelaide. They found the 1 ug of flagellin injected at birth and twice weekly thereafter until the rats were adults, produced firm tolerance (Nossal, Ada and Austin, 1965). A single large injection of 100 ug flagellin would induce tolerance but the tolerance was not long lasting; numerous injections of flagella into neonatal rats produced tolerance but large amounts of flagella were required. Antigen localization studies employing I^{125}-labelled flagellin revealed that tolerant rats handled the flagellar antigen in the same way as normal rats although no morphologic changes were observed in the lymph node draining the site of injection (Ada, Nossal and Pye, 1965). Cohen and Thorbecke (1964) demonstrated inhibition of morphologic changes in the lymph nodes draining the injection site of alum precipitated BSA in animals that were rendered tolerant to BSA at birth. Mitchell and Nossal (1966) have recently reported profound differences in the localization of flagellin, flagella and BSA in neonatal and adult rats. In the neonatal rat soluble antigen was diffusely distributed throughout the lymphoid tissue including the thymus. Phagocytosis in the neonatal rat was not obvious even when passive antibody was administered prior to the injection of antigen. Detectable antigen in the lymphoid tissue disappeared before tolerance was lost. In contrast adult animals readily localized antigen in the dendritic macrophages of lymphoid tissue (Nossal, Ada and Austin, 1964; Mitchell and Abbot, 1965). Whether complete tolerance was

induced in the preceding experiments is questionable as priming effects have been observed in tolerant rats (Nossal and Austin, 1966).

Induction of unresponsiveness in adult animals: high antigen dosage

The induction of unresponsiveness in adult animals by injecting large amounts of protein antigen stemmed directly from the induction of polysaccharide paralysis in mice (Felton and Ottinger, 1942; Felton, 1949). Dixon and Maurer (1955) induced unresponsiveness to BSA and human plasma in normal adult rabbits by the injection of over 20 gm of the respective proteins during a 1 - 2 month period. The tolerance lasted 3 - 4 months after the last injection of protein and in one case lasted over a year. Dresser (1962) found that a single injection of 150 mg BGG into adult mice induced complete tolerance; more or less than 150 mg BGG produced partial tolerance. It was also observed that if Freund's adjuvant (containing no BGG) was injected into mice simultaneously with 150 mg soluble BGG, tolerance was not induced. Sercarz and Coons (1959,1963) demonstrated that the tolerance induced in adult animals by high doses of polysaccharide or BSA was due to a lack of antibody forming cells rather than a "mopping-up" effect of antibody by excess antigen. They also reported that the tolerant state to BSA ends spontaneously after cessation of the antigen injections. The transfer of cells from tolerant mice to normal recipients did not lead to antibody

formation. Gitlin, Monckeberg and Craig (1958) had previously concluded that polysaccharide paralysis was due to specific inhibition of antibody synthesis.

An animal need not be a "virgin" with respect to previous contact with antigen in order to be rendered unresponsive to the same antigen. Dorner and Uhr (1964) were able to induce tolerance in 50 per cent of rabbits that had been previously sensitized to BSA; 200 mg BSA was injected every day into sensitized rabbits over a three week period producing a state of tolerance that persisted for several months. Dresser (1965) likewise rendered mice previously immunized to BGG partially tolerant to the same antigen by injecting 300 mg BGG followed by exponentially (x 2) decreasing amounts of BGG three times a week for three weeks. Recently Day and Farr (1966) produced protein-overloading "tolerance" to BSA in adult rabbits by conventional methods. The rabbits were rested for 139 days and rechallenged with 50 mg of I^{131}-labelled BSA. These animals were found to be unresponsive as judged by antigen elimination; however, low levels of antibody could be detected in the sera by the Farr technique after gel-filtration of the sera. Therefore these animals were immunologically suppressed rather than unresponsive to BSA. In light of these findings some past reports may have to be reviewed and future studies should employ primary measures of antibody detection if <u>complete</u> tolerance is to be studied.

One of the most definitive reports concerning the demarcation of factors governing the induction and maintenance of

tolerance in adult animals is the work of Mitchison (1964). It was shown that the degree of partial tolerance to BSA was related to the amount of BSA injected and the duration of the tolerance-inducing injections. Varying amounts of BSA were injected three times a week for 1 - 16 weeks. Two antigen dose zones of partial tolerance were observed: 1) the high doses of 5, 10 and 20 mg BSA produced almost complete suppression of the immune response to BSA after 8 - 14 weeks of treatment; 2) the low dose of 10 ug BSA induced partial tolerance in 1 - 4 weeks. Intermediary doses of BSA between 10 ug and 5 mg BSA, led to normal levels of antibody production. If the number of injections was reduced from three to one a week, the resulting partial tolerance was not as profound. If alum precipitated BSA was substituted for soluble BSA tolerance was not induced even after 16 weeks treatment. Above all, this paper clearly demonstrated that tolerance and sensitization can occur simultaneously in the same animal; the level of circulating antibody reflects the ratio of cells rendered tolerant and the number of cells sensitized to a particular antigen. Mitchison and co-workers have recently reported partial tolerance (as reflected in the delay of antibody production) to the hapten 4-hydroxy-3-iodo-5-nitrophenyl acetic acid (NIP) by the injection of 10 mg of either BSA-NIP or BGG-NIP as described above and later challenging with chicken globulin-NIP (Brownstone, Mitchison and Pitt-Rivers, 1966).

Induction of unresponsiveness in adult animals: X-irradiation and chemical treatment

Dixon and Maurer (1955) were among the first to demonstrate that whole body X-irradiation would render adult rabbits more susceptible to the induction of unresponsiveness. They found that if rabbits were injected with BSA or human plasma for 2 months after 400 r X-irradiation, the resulting unresponsive state would persist for 10 - 11 months; in contrast un-irradiated adult rabbits receiving only the protein injections were unresponsive for only 3 - 4 months. Nachtigal and Feldman (1963) were able to induce tolerance to BSA and HSA by injecting 20 mg of the respective antigens as long as 16 days after exposure to 550 r X-irradiation; the tolerance was maintained for over 90 days by periodic injections of BSA and HSA. They suggested that tolerance resulted from a depletion of lymphoid cells thus increasing the antigen/competent cell ratio. In contrast Rittenberg and Nelson (1963) found that a single injection of 10 mg BSA 24 hours after 400 r X-irradiation induced unresponsiveness but 100 mg BSA led only to a delayed immune response which occurred 14 - 24 days after the priming injection. Linscott and Weigle (1964) further demonstrated that tolerance to BSA lasting 2 months could be induced equally as well by injecting either 150 mg or 6 mg BSA on day 2 and day 7 following 550 r X-irradiation; injection of 0.5 mg BSA led to partial tolerance. Therefore it would appear that small amounts of antigen can induce tolerance in X-irradiated animals but larger amounts are needed to produce a prolonged

unresponsive state.

Mice may also be rendered unresponsive to antigens after whole body X-irradiation. Mitchison (1964) reported that the antibody response to BSA in mice exposed to 600 r X-irradiation was completely suppressed if greater than 10 ug BSA was injected three times a week after irradiation; 90 per cent inhibition was observed with 10 ug BSA and 50 per cent inhibition using 1 - 2 ug BSA. Brownstone, Mitchison and Pitt-Rivers (1966) recently reported that 600 r X-irradiation followed by BSA-NIP antigen would delay the onset of antibody production to the NIP residue upon later challenge with chicken globulin-NIP; partial tolerance with respect to the BSA carrier protein was induced. Dresser (1965) has found that the induction of partial tolerance to BGG in previously sensitized mice was facilitated by 500 - 600 r X-irradiation administered just prior to the first in a series of BGG injections; Dresser suggested X-irradiation was causing a generalized impairment of cellular function rather than increasing the antigen/competent cell ratio. Recently Staples, Gery and Waksman (1966) demonstrated that rats subjected to high doses of whole body X-irradiation with simultaneous spleen and thymus shielding did not become unresponsive to BGG if 20 mg BGG was injected either intraperitoneally or into the spleen; however, only 20 - 40 ug BGG was needed to induce unresponsiveness if the BGG was injected directly into the thymus.

Schwartz and Dameshek (1959) were able to induce tolerance

to HSA with the aid of 6-mercaptopurine (6-MP). The antigen was injected at the beginning of a 14 day treatment with 6-MP. The rabbits were challenged at one month intervals and were found to be tolerant to HSA; specificity of the tolerant state was demonstrated as the tolerant rabbits could elicit a normal immune response to BGG one month after the cessation of the 6-MP treatment. It was shown later that unresponsiveness facilitated by 6-MP was antigen dependent with respect to dosage, i.e., greater than 66 mg of BSA had to be injected in order to regularly induce a tolerant state (Schwartz and Dameshek, 1963). Nachtigal and Feldman (1963) also demonstrated lasting suppression to BSA and HSA in rabbits by injecting the antigen together with 6-MP. Maguire and Maibach (1961) rendered guinea pigs refractory to the induction of systemic anaphylaxis to EA by prior treatment of the animals with nitrogen mustard and EA. Salvin and Smith (1964) induced unresponsiveness to BGG and BGG-p-azobenzoate by intraperitoneal injection of 20 - 40 mg of the respective antigen plus 80 mg of cyclophosphamide; tolerance was specific as the guinea pigs were able to elicit an immune response to other antigens upon later challenge. Dresser (1965) attempted without success to facilitate the induction of tolerance to BGG in mice previously sensitized to BGG by a combination of 6-MP and high doses of BGG; the lack of success was not surprising as Schwartz and Dameshek (1958) found that 6-MP did not affect the primary antibody response if the drug was administered some days after the injection of the antigen. Acriflavine has been found to

induce partial tolerance to BSA and BGG when the antigens were injected at the beginning of a 14 day treatment with the drug (Samuelson, Stewart, Kraft and Farr, 1965; Samuelson, Kraft and Farr, 1965). More recently Farr and co-workers have reported that the induction of tolerance to BSA facilitated by acriflavine was most profound when small doses of BSA (i.e., 1 mg BSA) were administered as compared with the injection of larger doses (i.e., 10,100 or 1,000 mg BSA); therefore it would seem that acriflavine suppression may be potentiated by mechanisms different from X-irradiation or 6-MP suppression (Kraft, Samuelson and Farr, 1967).

Induction of unresponsiveness in adult animals: low antigen dosage and/or aggregate-free antigen

One of the most important observations in the study of immunological tolerance stemmed from the work of Dresser (1962a). He found that relatively small amounts of centrifuged, aggregate-free BGG injected intraperitoneally, rendered mice unresponsive to later challenge with BGG in Freund's adjuvant; the minimum amount of BGG required was 50 - 200 ug, although 2,000 ug was far superior. Three to four days were necessary to elapse between the tolerance-inducing injection and the challenging injection in Freund's adjuvant in order to induce a profound degree of tolerance. Claman (1963) confirmed Dresser's earlier findings; it was also observed that, if 100 ug of S. typhosa endotoxin was injected intravenously two hours after the intraperitoneal injection of

aggregate-free BGG, the mice did not become tolerant and elicited a normal immune response to BGG. Recently Claman and Bronsky (1965) also demonstrated that actinomycin D, given prior to the injection of a normally tolerogenic dose of centrifuged BGG, blocked the induction of tolerance. Battisto and Miller (1962) produced unresponsiveness in adult guinea pigs by injections of from 5 - 500 ug of aggregate-free BGG into the mesenteric vein.

Dietrich and Weigle (1964) rendered mice tolerant to human and turkey gamma globulin and partially tolerant to BSA; a two dose zone of partial tolerance to BSA was noted, being quite similar to the data of Mitchison (1964). Transfer of lymphoid cells from mice tolerant to human or turkey gamma globulin into X-irradiated recipients did not lead to antibody production. Taylor (1964) produced unresponsiveness in adult mice by numerous injections of 250 ug BSA. Mitchison termed this type of tolerance as "cumulative" and clearly demonstrated the relation between the antigen dose and the duration of antigen injections upon the resulting partial tolerance; it was found that 10 ug BSA injected three times a week induced partial tolerance in 1 - 3 weeks. Rowley and Fitch (1964) reported that low dose tolerance could be induced in adult rats to sheep erythrocytes; although there was no detectable circulating antibody in the "tolerant" rats, there was an increase (10-100 fold) in the number of plaque forming cells in the spleens of these animals.

Unresponsiveness to leporine (hare) gamma globulin but

not horse gamma globulin has been induced in adult rabbits from a single injection of centrifuged, aggregate-free antigen (Dresser and Gowland, 1964). Frei, Benacerraf and Thorbecke (1965) induced tolerance or suppression to BSA after injection of 6 - 8 mg of "in vivo filtered" BSA; one third of the rabbits were tolerant to BSA upon challenge 21 and 35 days later. Biro and García (1965) induced tolerance to HGG by injecting 5 mg of the centrifuged, aggregate-free HGG. If the aggregate-free HGG was heated to 63°C for 15 minutes, it would initiate a normal immune response. The work also demonstrated that the induction period for a profound degree of tolerance was greater than 24 hours and less than 7 days.

Termination of the unresponsive state

Tolerance may be abrogated by stopping the injection of antigen to which the animal is unresponsive. Restoration may occur relatively quickly or may take many months; the variations which have been observed could be explained in part to different degrees of tolerance to various antigens. Spontaneous development of antibody production in animals previously tolerant without further injection of antigen has been observed; this phenomenon has been termed "overshoot". Terres and Hughes (1959) noted overshoot in mice rendered tolerant to BSA at birth; Thorbecke, Siskind and Goldberger (1961) also noted this reaction to BGG. Paralysis to pneumococcal polysaccharide may spontaneously end with the production of immunity (Siskind, Paterson and Thomas, 1963). Antibody

production was observed in mice tolerant to BSA and polysaccharide a few weeks after circulating antigen could no longer be detected (Sercarz and Coons, 1959, 1963). The preceding observations indicated that cells lose their tolerant state after depletion of antigen. However, Mitchison (1965) has recently observed that overshoot occurs to a greater degree in younger animals; he interpreted this finding as an indication that newly formed competent cells could not be rendered tolerant in such a situation and that relics of antigen remaining in the body could sensitize these competent cells. Another interesting observation concerning the nature of the immune response following the natural termination of tolerance is that the recovery from tolerance occurs "piecemeal" with respect to the antigen mosaic (Humphrey, 1964a); therefore it is likely that although tolerance may have terminated to one area of a protein antigen, a tolerant state may persist to other portions of the antigen molecule.

The injection of proteins of related specificity to the tolerated antigen can often terminate an unresponsive state to some degree. Curtin (1959) reported abrogation of tolerance in 4 out of 15 rabbits tolerant to Bence-Jones proteins by injecting myeloma protein in Freund's adjuvant. Weigle (1961) terminated neonatal-induced tolerance to BSA by injecting HSA in the tolerant adult rabbits; albumins that were antigenically more closely related to BSA, such as pig albumin or sheep albumin, did not appreciably abrogate tolerance to BSA. An

interesting termination of tolerance to self antigens was reported by Bussard and Hannoun (1962); it was shown that the injection of sheep erythrocytes into adult rabbits resulted in the in vitro production of antibodies specific for rabbit erythrocytes. Autoimmunity to thyroglobulin has been demonstrated by the injection of heterologous thyroglobulin (Weigle, 1965). Weigle (1964) has reported that abrogation of tolerance to BSA in rabbits by injection of HSA resulted only in the formation of antibodies which were directed against shared antigenic determinants. It was also observed (Weigle, 1964a) that the injection of HSA not only abrogated tolerance to BSA but resulted in a secondary type response to HSA; often when these rabbits were re-injected with BSA, a state of tolerance was once again established. The injection of BSA together with HSA does not terminate BSA tolerance in adult rabbits (Weigle, 1964a; Humphrey, 1964). Weigle (1964a) has also reported the termination of BGG tolerance in rabbits by the injection of HGG. Mitchison (1964) as in previous reports abrogated BSA tolerance in adult mice by injection of HSA; however, Mitchison noted that the degree of reactivation was related to the degree of partial tolerance. The complexity of the mechanisms involved in the abrogation of tolerance with related proteins is difficult to elucidate; Day and Farr (1966) have recently discussed the question of shared antigenic determinants and the production of antibodies with varying affinities to the various antigenic determinants. The ultimate answer may lie in the fact that many of the studies

actually are terminating a partially tolerant state rather than a completely tolerant state.

Chemically altered antigens may accelerate the termination of tolerance; Cinader (1963) has reviewed this topic. Cinader and Dubert (1955,1956) were able to terminate tolerance to HSA in a few instances by injecting sulphanil-HSA. Weigle (1962) studied the ability of a wide variety of modified proteins to abrogate tolerance; BGG tolerance was easier to terminate than BSA tolerance, arsanil-BGG, sulphanil-BGG or arsanil-sulphanil-BGG all abrogating tolerance to BGG. Arsanil-sulphanil-BSA was the only modified derivative of BSA that would consistently break tolerance to BSA. In some cases rabbits that had received azo-BSA derivatives would again become tolerant to BSA upon further injection of BSA (Weigle, 1964). Weigle (1965) induced unresponsiveness to picryl-BSA by neonatal injection of the complex; the adult animals surprisingly responded to picryl-horse gamma globulin with antibodies specific for the picryl residue. Tolerance to BSA was not broken nor did the injection of picryl-rabbit albumin or DNP-BSA abrogate tolerance to BSA or give rise to antibodies reacting with the picryl group. These observations seem to indicate that tolerance is directed towards a relatively large area of the antigen molecule and that tolerance to haptens may be carrier specific.

Nachtigal and Feldman (1964) reported that immunization of rabbits with sulphanil-rabbit albumin led to the formation of antibodies with HSA specificity; it was suggested that hidden

determinants in the rabbit albumin were exposed during the coupling reaction which cross-reacted with HSA. Tolerance to HSA in rabbits could be completely broken by injecting sulphanil-HSA followed by injections of native HSA (Nachtigal, Eschel-Zussman and Feldman, 1965); these results conflict with those of Weigle. It is interesting that Weigle induced tolerance by neonatal injection of 500 mg of protein thus producing a profound degree of tolerance; Nachtigal and co-workers on the other hand induced tolerance in adult rabbits after 550 r X-irradiation and perhaps did not induce a profound tolerant state. Moreover, Linscott and Weigle (1965) found that, although arsanil-sulphanil-BSA terminated tolerance to BSA, the antibodies were more specific for the BSA conjugate even after repeated injections of BSA over a six month period. One other interesting observation was that if HSA was injected together with sulphanil-HSA into tolerant rabbits, the tolerant state was not terminated (Nachtigal, Eschel-Zussman and Feldman, 1965). Attempts to break tolerance to rabbit albumin by the injection of sulphanil-rabbit albumin also failed. Weigle (1965) on the other hand abrogated the natural tolerance to rabbit thyroglobulin by the injection of arsanil-sulphanil-rabbit thyroglobulin; this observation could be explained simply by low levels of thyroglobulin in the circulation; in this situation tolerance might be more difficult to maintain.

Other factors influencing abrogation and maintenance of unresponsiveness

Lymphoid cells from normal and immunized rabbits sensitized to BSA did not break tolerance when injected in rabbits tolerant to BSA (Smith and Bridges, 1958). Passive antibody and/or X-irradiation had no effect upon the termination of tolerance to BSA in rabbits (Weigle, 1964). "Adoptive tolerance" has been demonstrated in a number of reports. Smith and Bridges (1959) injected lymphoid cells from rabbits tolerant to BSA into X-irradiated normal rabbits and failed to demonstrate antibody production before the onset of homograft rejection. The transfer of lymph node and spleen cells from inbred rats tolerant to sheep erythrocytes into lethally irradiated recipients produced adoptive tolerance lasting up to six months (Stastny, 1964). Dietrich and Weigle (1964) demonstrated adoptive tolerance to human and turkey gamma globulin in mice. Isakovic, Smith and Waksman (1965) produced adoptive tolerance to BGG lasting 3 - 6 weeks by implantation of thymus grafts from rats tolerant to BGG. Relevant to the above reports, Dutton (1964) was unable to demonstrate any proliferative effects when lymphoid cells from rabbits tolerant to either EA or BSA were incubated in vitro with EA or BSA.

Adult thymectomy has been demonstrated to prolong neonatal induced tolerance to BGG (Claman and Talmage, 1963). On the other hand X-irradiation hastens the breakdown of BGG and BSA tolerance in mice (Claman and McDonald, 1964; Mitchison, 1965); however, X-irradiation and thymectomy cancel their opposing

effects (Claman and McDonald, 1964). Taylor (1964) has demonstrated prolonged suppression to BSA by thymectomy. Weigle (1964) on the other hand reported no beneficial effect of adult thymectomy in tolerant rabbits; this could be explained either because the rabbits initially possessed a profound tolerance or that the appendix of the rabbit may act as a central lymphoid organ causing "peripheralization" of lymphoid cells in the absence of the thymus. Mitchison (1965) has recently shown that the speed of recovery from tolerance is a function of the age of the animal, younger animals recovering more rapidly. This together with the above findings seem to indicate that cell turnover is an important factor in the natural breakdown of acquired immunological tolerance.

Objectives of Proposed Research

The basic objective of the present research has been to elucidate the mechanisms involved in the initiation of the antibody response. Three areas of study being, in the author's view, relevant to such a study were 1) the change of the relative binding affinity of antibody after immunization, 2) the adjuvant effects of various substances and 3) the induction of immunological unresponsiveness vis-a-vis immunological reactivity. Any insight into areas 1 and 2 would be likely to provide direct information which could then be applied to area 3. It therefore seemed desirable to study some of the factors which completely suppress, partially

suppress or augment antibody production.

There were two main ways of approaching this problem. The first was the chemical or physical modification of the antigen and the second whereby the cellular processes concerned with the uptake and processing of antigen were altered. Chemical modification of the antigen was excluded immediately as this would be likely to expose and/or add new antigenic determinants. The physical state of the antigen could, however, be controlled; the degree of aggregation of a protein had been previously shown to have dramatic effects upon antibody production (Dresser, 1962a; Claman, 1963; Frei, Benacerraf and Thorbecke, 1965; Biro and García, 1965). This model in which an antigen can act as a powerful stimulus to initiate antibody production or in a different form to induce an unresponsive or suppressed state provided a means whereby the role of the cellular mechanisms of the lymphoreticular system could be studied by suitable modification of their activity.

A wide variety of substances ranging from simple chemicals to complex bacterial products had been shown to have profound effects upon lymphoreticular cells. Dr J.G. Howard suggested that Corynebacterium parvum might provide the necessary stimulation of lymphoreticular tissue. Corynebacterium parvum had been shown to have one of the most powerful stimulatory effects upon lymphoreticular cells, causing hepatosplenomegaly, marked increase in phagocytic activity (Halpern, Prévot, Biozzi, Stiffel, Mouton, Morard, Bouthillier and Decreusefond, 1964)

and an adjuvant effect on antibody production (Neveu, Branellac and Biozzi, 1964; Howard, personal communication). Preliminary studies revealed that in moderate doses, heat-killed suspensions of Corynebacterium parvum were not toxic to newborn rabbits; in fact fewer neonatal deaths and an increased weight gain was found in treated compared to untreated animals (Pinckard and Weir, unpublished results). This material, therefore, in combining powerful stimulatory effects on the lymphoreticular tissues with lack of toxic side effects appeared to be eminently suitable.

As will be discussed at greater length presently, it is now well established that most of the classical serological tests, which measure secondary effects of antibody-antigen interaction, select or are more sensitive towards certain populations of antibody molecules depending on the antigen being studied. The present study was therefore designed to assess the entire humoral immune response by measuring the antigen-binding capacity as determined by the ammonium sulphate method of Farr (1958); for this reason bovine serum albumin was chosen as the antigen. Preliminary experiments indicated that centrifuged, aggregate-free BSA rendered a large percentage of rabbits tolerant or partially tolerant to BSA upon later challenge. The Farr technique enabled the estimation not only of quantitative differences in antibody production but also changes in the relative binding affinity of the antibodies produced.

The Detection and Characterization of Humoral Antibody Production

Since the birth of immunology in the late 1800s the field has been concerned to a great degree with the protective aspects of innate and acquired immunity. Many of the procedures which were developed during this period of infancy are still being extensively employed today; some examples of tests developed around 1900 are the precipitin reaction, bacteriolytic and bacteriocidal tests, complement fixation, opsonization, neutralization of toxins and agglutination reactions. It is remarkable that so many fundamental principles and theories emerged during these early periods; the techniques available then could be described as rudimentary, yet we find that a large segment of contemporary research is studying many of the same problems and is striving to elucidate the same unanswered questions. The advantages we possess today over and above hindsight and our enlarged knowledge are the technological advances in methodology which have provided more sensitive and more accurate means to assess the humoral antibody response. Our main problem today is not to continue to assess the pragmatic effects of innate and acquired immunity, as has been done for the last 80 years, but to elucidate the mechanisms of antibody formation so that we are able to control this process in the medical sphere.

The interaction of antigen and antibody can be described as a two stage process. Initially the antigen and antibody

molecules combine chemically to form an antigen-antibody complex. The kinetics of this interaction have been known for a number of years. In the early 1930s Marrack and Smith (1932) and Haurowitz and Breinl (1933) independently developed the technique of equilibrium dialysis which enabled the determination of the association constant between a hapten molecule and its specific antibody molecule. The technique was later modified by Eisen and Karush (1949) into a procedure which today is of primary importance in the elucidation of the antibody combining site (see Pinckard and Weir, 1967). A technique such as equilibrium dialysis which measures or is solely dependent upon the primary interaction of antigen and antibody can be said to be a primary measure of antibody production. After the primary interaction between antigen and antibody, a series of complex, often obscure reactions can occur depending upon the type and quality of antibody present and in many instances is also influenced by the antigen and environmental factors. The agglutination of bacteria, viruses or erythrocytes or precipitation of antigen-antibody complexes are examples of such reactions. This type of reaction may be called a secondary measure of antibody production. Finally, even more complex reactions can occur which are even more obscure such as the lysis of an erythrocyte, killing of a bacteria, survival of an animal from infection or the in vivo elimination of the antigen-antibody complex. These reactions may be classified as tertiary measures of antibody production. The most important point which must be realized is that

although secondary and tertiary measures of antibody production reflect primary interaction of antibody and antigen (i.e., the presence of specific antibody), primary interaction of antibody and antigen does not necessarily result in secondary or tertiary manifestations. Therefore if no secondary or tertiary reactions are noted with an antiserum, this does not mean that there is no antibody present; secondly, one can also predict that the quantitation of antibody by secondary or tertiary measures may also be very unreliable.

The above description, although it has been known for years, is often neglected. During the early 1900s immunologists believed that each secondary or tertiary reaction to a specific antigen was initiated by a distinct antibody. Zinnser (1921) realized at this time that, although quantitatively there were differences in the secondary or tertiary reactions, antibody was specific for its antigen and proposed the unitarian theory of antibody. Zinnser wrote, "In appraising such objections, we must not forget that agglutination and precipitation are actually secondary phenomena, after the union of antigen and antibody has taken place, and are dependent upon a great many environmental factors which may not, to the same degree, influence phenomena in which alexin, the leucocyte or the body cells of animals are involved." Although the unitarian theory may still be correct in a modified form, it is only true with respect to the primary interaction of antigen and antibody and not to secondary or tertiary reactions. The unitarian theory in its entirety, including the secondary and

tertiary portions, is unfortunately still accepted by some workers.

Today many investigators are studying "mechanisms of antibody formation" or "mechanisms of immunological tolerance"; it is therefore of crucial importance to use methods which will detect all antibody produced. As will be discussed at greater length, the only way this can be done properly is by employing only those tests which are solely dependent upon the primary interaction of antigen and antibody. Secondary and/or tertiary measures can only be used if account is taken of their limitations and the results related to the particular type of antibody taking part in the secondary or tertiary manifestations observed. Otherwise, as so often occurs even in the current literature, misleading interpretations will be made. Boyden (1959) has recently suggested criteria for the "utopian serological test" which is relevant here.

1. The test should detect specific antibodies in very small amounts.
2. It should measure the primary interaction of antibody and antigen and it should not depend upon secondary reactions which only take place in certain cases.
3. It should be capable of providing quantitative data concerning the amount of antibody present and also concerning its "avidity".
4. It should tell something about the biological properties of the antibody which it detects, being able, for example, to distinguish between precipitating, non-precipitating

and skin-sensitizing antibodies, etc.

5. It should be applicable to any antibody-antigen system.

6. It should be simple.

The present author, although agreeing in principle with the above criteria, believes that the first three points are by far the most important aspects of a good test for antibody.

Farr (1958) pointed out the dangers of using tests which measure secondary interaction and demonstrated antibodies which differed with respect to precipitability during the immune response; he also cited previous reports supporting this hypothesis. It had been shown that some antibody to soluble protein antigens would co-precipitate but would not precipitate on its own (Heidelberger and Kendal, 1935). Anti-BSA antibody which is produced at different stages of the immune response has differing abilities to precipitate BSA (Heidelberger and Kendall, 1935; Heidelberger, Freffers and Mayer, 1940; Talmage and Maurer, 1953). The fixation of complement was found not to correlate with the ability of an antiserum to precipitate (Wallace, Osler and Mayer, 1950). It had also been shown that precipitation may be affected by non-specific factors such as dilution and the presence of complement (Maurer and Talmage, 1953; Weigle and Maurer, 1957).

The heterogeneity of antibody in secondary and tertiary reactions has also been noted in haemolysin production (Stelos, 1956; Talmage, Freter and Taliaferro, 1956; Goodman and Masaitis, 1950). Fiset (1962) reported non-precipitating

antibodies to ovalbumin and BSA which did not give a positive passive haemagglutination test. The ability of guinea pig antibodies of the γ_1 and γ_2 antibody to yield positive PCA and complement fixation respectively but not simultaneously led Block, Kourilsky, Ovary and Benacerraf (1963) to caution about using a single antibody test when studying the immune response. It was also shown in this study that the γ_2 antibody was far more effective in opsonization. Differences in biologic and serologic reactions between toxins and antitoxins have been observed (Raynaud, 1959; Robbins, 1965). Recently Schwartz and Dameshek (1963) reported conflicting results using passive haemagglutination, immune clearance and quantitative precipitin tests when studying drug induced immunological suppression. Greenbury, Moore and Nunn (1963) reported that rabbit 19S antibody was 750 times more efficient as 7S antibody in agglutinating human group A erythrocytes. Robbins, Kenny and Suter (1965) have likewise demonstrated the apparent superiority of 19S antibody in certain secondary and tertiary tests; for example it was found that 19S antibody was 22 times more effective than 7S in agglutination, 120 times more effective in complement fixation and 500 - 1,000 times more effective in opsonization than equimolar concentrations of 7S antibody.

Finally it is necessary to mention more recent work which has assessed the antibody response using both primary and secondary measures for detecting antibody. Specifically purified chicken anti-BSA antibody obtained from 6 day primary

sera, although found to be 2-mercaptoenthanol sensitive and giving a positive passive haemagglutination reaction with only the 19S fraction, was found to be predominantly 7S when characterized by analytic ultracentrifugation (Pinckard and Benedict, unpublished results). It was further demonstrated that 6 day primary chicken anti-BSA, anti-HSA and anti-p-azo-benzoate antibodies were predominantly 7S as determined by the Farr technique, radioimmunoelectrophoresis and equilibrium dialysis; passive haemagglutination on the other hand revealed antibody activity only of the 19S class (Dreesman, Larson, Pinckard, Groyon and Benedict, 1965). Rabbit anti-BSA antibodies have been shown to vary in ability to precipitate and to give positive passive haemagglutination reactions when normalized to the Farr technique (Grey, 1964; Benedict, 1965). Human sera have been shown to differ widely in secondary and tertiary tests for the detection of anti-BSA antibodies when compared to primary binding of antibody (Minden, Reid and Farr, 1966). Radioimmunoelectrophoresis has been shown to detect antibody which is not normally detected using secondary measures (Freeman and Stavitsky, 1965). Rabbits showing no immune elimination possess antibody which may actually prolong retention of antigen (Day and Farr, 1966). Using an anti-globulin (Coombs reagent) test Allemeier, Robbins and Smith (1966) have demonstrated antibody of the 7S class to the somatic antigen of Salmonella organisms which up to that time normally went undetected. With the number of published reports concerning the limitations of tests which are dependent

upon secondary and/or tertiary reactions, there can be no justification for using these procedures unless the published data specifically draws attention to the pitfalls of such methods.

The primary measures of antibody production should not by any means be considered as absolute. Many are limited in sensitivity and are not entirely without error. Until improved and more sensitive tests are developed, however, we must use the existing ones with understanding and valid criticism. The continuation of the use of secondary and tertiary measures of antibody production, especially when studying "mechanisms of antibody formation" and "mechanisms of immunological tolerance", in light of our present knowledge is inexcusable and will most likely lead not to the elucidation of concepts but to further confusion. Table 1.1 lists a number of antibody tests giving pertinent data as to the type of measure involved and whether the test is quantitative and/or qualitative.

Table 1.1

Primary, Secondary, and Tertiary Antibody Tests

Antibody Test	Primary	Secondary	Tertiary
Complement Fixation			X
Bacterial Immobilization			X
Bacteriocidal			X
Bacteriolytic			X
Immune Clearance			X
Opsonization			X
Passive Cutaneous Anaphylaxis			X
Systemic Anaphylaxis			X
Toxin Neutralization			X
Viral Neutralization			X
Survival Rate			X
Bacterial Agglutination		X	
Erythocyte Agglutination		X	
Passive Haemagglutination		X	
Quantitative Precipitin		X	
Gel Diffusion		X	
Fluorescent Antibody	X		
Equilibrium Dialysis	X		
Fluorescent Quenching	X		
Farr Technique	X		
Fluorescent Polarization	X		
Radioimmunoelectrophoresis	X		
Coombs Reagent Tests	X		

MATERIALS AND METHODS

Bleeding, Injecting and Care of Animals

Explanatory note

Although the care and handling of experimental animals may be considered subordinate in importance to other experimental procedures, it is felt that good technique when working with experimental animals is of paramount importance. In any type of scientific investigation of "cause and effect" relationships one must control the many variables which might lead to false conclusions. The animal is one such important variable. The individual genetic variations in a species can be virtually eliminated by inbreeding but the variables which may result from the care and handling of experimental animals can be minimized only by the use of standardized techniques. Experimental animals must be fed a proper diet which is uniform and controlled and they must be healthy and free from disease, clinical or subclinical. Psychological disturbances must be avoided because this could lead to physiological alterations which might influence *in vivo* mechanisms.

Animals

New Zealand White (NZW) rabbits were used throughout this study. Adult animals of both sexes were used, and were from 3 - 4 months of age and weighed between 2.0 Kg and 3.5 Kg., unless otherwise stated.

Feeding and Housing

The rabbits were fed on a standard rabbit pellet feed.

They were given fresh water daily and fresh greens (either cabbage or cauliflower) twice a week. The rabbits were kept in separate cages with approximately 2 square feet of floor space; the bottoms of the cages were 1/2 inch wire mesh. The cages were cleaned twice each week. Before a rabbit was placed into a new cage, the cage was thoroughly washed and autoclaved to minimize cross-infection.

Breeding

The doe was from 6 to 18 months of age. A special breeding cage was used and had 4 square feet of floor space. The doe was allowed to become accustomed to the breeding cage for two to three weeks. During this time the experimenter fed the doe in order that she became accustomed to the presence of human scent. The does were fed as described above but had additional bran meal. The buck was placed into the breeding cage with the doe and was allowed to remain for one week after which time he was removed. Hay was then placed into the cage for nesting material. From 3 - 6 hours were allowed to elapse after the babies were born before handling. The babies were separated from their mother when they were six weeks of age.

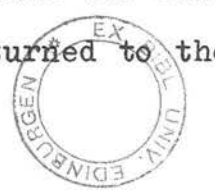
Injecting

The rabbits were placed into a rabbit box and the ear was washed with 70% ethyl alcohol. The hair covering the marginal ear vein was pulled out; this procedure irritated the area and caused dilatation of the vein. The sterile solution was then injected through a 25 gauge needle into the marginal ear vein.

The success of the injection was noted by the loss of colour in the vein as the solution was injected. The needle was then quickly withdrawn and the vein occluded for 30 seconds in order to avoid the formation of a haematoma.

Bleeding

Two methods of bleeding were employed. When only minute quantities of blood were required the marginal ear vein was washed with 70% ethyl alcohol and a longitudinal cut made with a scalpel. After the desired quantity of blood was taken the vein was occluded until no further bleeding occurred. The other method of bleeding was by cardiac puncture. Although one person can easily perform this procedure, two people were generally employed as this greatly expedited the procedure. The rabbit was restrained by placing it on its back on a rabbit board. The entire thoracic area was thoroughly washed with 70% ethyl alcohol. A 19 gauge needle was used to withdraw the blood from the heart. The needle was inserted between the third and fourth rib until the heart could be felt beating against the needle. With a quick thrust the needle was inserted into the heart. After the blood had been drawn the needle was quickly withdrawn from the chest. The method of cardiac puncture has been found to be a highly efficient method for collecting blood and caused minimal psychological trauma to the rabbit. The time taken to bleed one rabbit by this method was 40 seconds from the time the rabbit was taken from its cage until the time it was returned to the cage.



Collection of sera

After the blood had been drawn, it was allowed to clot for 30 minutes in a 37°C water bath, rimmed and allowed to incubate for a further 15 - 20 minutes. The blood was placed at 4°C overnight. After removal of the clot the sera was centrifuged at 2,000 R.P.M. (1,500 R.C.F.) for 15 minutes. The sera was decanted into tubes and frozen at -20°C until needed.

Quantitative and Differential White Blood Cell Counts

Explanatory note

The quantitative and differential white blood cell counting procedures are used simultaneously in order to obtain a comprehensive picture of the blood leucocyte levels. The quantitative count gives the total number of white blood cells per cubic millimeter while the differential white count gives the percentage of each type of white blood cell present in the blood. It is then possible, therefore, to calculate the number of each type of white blood cell per cubic millimeter of blood. Improved Neubauer counting chambers were employed in this study for the quantitative white cell counts and blood smears were stained with Leishman's stain for the differential white cell counts.

Materials

1. Leishman's stain:

150 mg Leishman stain - ground to a fine powder;

100 ml methyl alcohol.

The Leishman stain was mixed with the methyl alcohol and allowed to incubate at 37°C overnight in a sealed container. The solution was then filtered and stored at room temperature.

2. Distilled water, pH 7.0.
3. 1% acetic acid.
4. Improved Neubauer counting chambers.

Procedure

Quantitative white cell counts: 0.2 ml of blood was collected from the marginal ear vein and immediately transferred into 3.8 ml of 1% acetic acid and was mixed thoroughly. The Neubauer counting chamber was cleaned with distilled water and wiped completely dry. The cover slip was then placed on the counting chamber and seated until Newton's rings formed. The lysed blood described above was allowed to flow into the counting chamber from a Pasteur pipette, care being taken not to permit any overflowing. The number of white cells in each of the four 1.0 mm squares was counted under low power with the aid of a hand counter. Calculation of the number of white cells/cubic mm: the depth of the Neubauer counting chamber is 0.1 mm and therefore a total volume of 0.4 cubic mm was counted; the initial dilution of blood was 1/20 and thus

the number of white blood cells/cubic mm of blood is equal to the number of cells counted times 50.

Differential white cell counts: A drop of blood from the marginal ear vein was placed on a clean glass microscope slide and a blood smear was made. When the blood film had air dried, 15 drops of Leishman's stain were applied with a Pasteur pipette and allowed to incubate at room temperature for 60 seconds. After this time 15 drops of distilled water, pH 7.0, were applied to the slide and mixed by gentle rocking. Fifteen minutes later the slide was washed in distilled water for 1 minute and air dried. The stained slides were viewed under a magnification of 1,000 X using the oil-immersion objective. The numbers of large and small lymphocytes, monocytes and polymorphonuclear leucocytes were counted; the total number of cells counted was not less than 100. The percentage of each cell type was then determined.

Determination of the Phagocytic Activity of the R.E.S.

Introductory note

The use of colloidal carbon particles has been used extensively for measuring the phagocytic activity of the reticulo-endothelial system (R.E.S.). In earlier work india ink was employed but the results were influenced by the release of thromboplastin caused by shellac in the india ink (Halpern, Benacerraf and Biozzi, 1953). By employing a highly purified colloidal carbon preparation, Biozzi,

Benacerraf and Halpern (1953) were able to quantitatively demonstrate specific phagocytic activity, independent of toxic side effects, and establish mathematical equations describing the clearance of carbon from the blood.

There are three factors which govern the phagocytic activity: 1) particle size of the carbon suspension, 2) amount of carbon injected per 100 gm body weight and 3) the degree of saturation of the phagocytic cells with carbon. By employing carbon particles of 250 $\mathring{A}$ in diameter Biozzi et al. (1953) found that the phagocytic activity could be described by the following equation,

$$K = (\log C_o - \log C)/T \qquad 1.$$

where C is the concentration of carbon at time T and C_o is the concentration of carbon in the blood immediately after injection and K is the phagocytic index. By transforming equation 1. it can be seen that

$$\log C = - KT + \log C_o \qquad 2.$$

A linear relationship can be found between the log concentration of carbon remaining in the blood at time T and the log of the initial concentration of carbon. The phagocytic index K can be determined from the slope of the line described in equation 2. by the slope-intercept form, i.e.,

$$y = \log C$$

$$x = T$$

$$\text{slope} = - K$$

$$\text{y-intercept} = \log C_o$$

Although K may be used to compare the phagocytic indices

among groups of animals, variations in the spleen and liver weight will result in variations of the K values. It has therefore been proposed that a corrected phagocytic index be used (Biozzi et al., 1953). The corrected phagocytic index is described in equation 3,

$$\alpha = (B.W./(L.W. + S.W.))K^{1/3} \qquad 3.$$

where α is the corrected phagocytic index, K is the phagocytic index calculated from equation 2, B.W. is the total body weight of the animal, L.W. is the liver weight and S.W. is the spleen weight. The following procedure to calculate K and α is slightly modified from the method described by Biozzi et al. (1953).

Materials

1. Colloidal carbon, 110 mg/ml
2. 4% gelatin in distilled water
3. 0.1% Na_2CO_3

Procedure

Preparation of carbon in 2% gelatin: The colloidal carbon was centrifuged at 3,000 R.P.M. for 30 minutes and the top 3/4 of the suspension was drawn off. One volume of 4% gelatin (at 56°C) was added to one volume of the centrifuged colloidal carbon and the resulting mixture was incubated at 56°C until injected.

Clearance experiment: The rabbits were placed in a restraining box and the marginal ear vein of the right ear was

cut with a scalpel and occluded with a piece of cotton wool soaked in heparin. Between 4 - 6 ml of the carbon-gelatin suspension was then injected into the marginal ear vein of the left ear; each rabbit received 8 mg of carbon per 100 gm of body weight. At frequent intervals after the injection of carbon, 0.20 ml of blood was collected from the right ear and was immediately placed in 4 ml of 0.1% Na_2CO_3. The periodic bleedings were continued until the blood appeared devoid of carbon. The lysed blood samples were then read on a Unicam Sp 1300 spectrophotometer using filter # 6 (620 mu) and the O.D. values corrected for the slight absorbance due to haemoglobin.

Calculation: The data were plotted as described by equation 2, the log O.D. being the y-axis and the time in minutes the x-axis. The best straight line was drawn through the points using the method of least squares. The phagocytic index K was determined as the slope of the plot times minus one. The corrected phagocytic index was determined as shown in equation 3.

Preparation of Corynebacterium parvum

Explanatory note

Corynebacterium parvum strain 10387 was originally obtained from the National Collection of Type Cultures, Colindale, London. The organism was grown anaerobically in a special cooked-meat broth containing 1% glucose. To ensure

that the cooked-meat broth was devoid of bovine antigens, which if present might have sensitized the animals to BSA, it was prepared from sheep muscle. Great care was taken to ensure purity of the culture and uniform growth of the organism.

Materials

1. 500 g sheep heart
2. 500 g lean sheep meat
3. 1 N NaOH
4. NaCl
5. Seitz-filtered sterile 10% glucose

Procedure

Cultivation media: 500 g of sheep heart, trimmed free of all fat and connective tissue, was minced and boiled for 20 minutes with 500 ml of distilled water and 1.5 ml of 1 N NaOH. The resulting suspension was filtered through muslin and the solid material was blotted dry. The minced cooked heart was then distributed in 100-ml and 500-ml bottles to a depth of 1 - 2 inches and this was overlaid with a sheep meat infusion broth until each bottle was 4/5ths full. The infusion broth was prepared from 500 g of lean sheep meat which was minced and mixed with 1 litre of distilled water containing 5 g NaCl. This mixture was incubated for 24 hours at 4°C. It was then boiled for 15 minutes, filtered through muslin and then filter paper. The pH of the filtrate was then adjusted to 7.4 with 1 N NaOH. After the bottles were

filled with the desired amounts of minced cooked heart and infusion broth, they were sealed and autoclaved at 121°C at 15 lbs pressure for 15 minutes.

Cultivation of C. parvum: Just prior to inoculation with the stock culture, each bottle of culture medium was enriched with sufficient Seitz-filtered sterile 10% glucose solution to bring the glucose concentration to 1%. The bottle was then sealed loosely with its screw cap and steamed for 20 minutes which ensured anaerobic conditions. A 100-ml bottle of the cooked-meat broth was inoculated with C. parvum and incubated anaerobically at 37°C for 48 hours. The growth of this starter culture was then checked for purity by examination of Gram-stained smears and by subculture on blood agar enriched with glucose. Meanwhile a 25-ml volume of the starter culture was inoculated into each of four 500-ml bottles of culture medium and these were incubated without shaking for 3 days at 37°C under aerobic conditions.

Preparation of C. parvum for injection: The C. parvum cultures were filtered through 8 layers of muslin in order to remove any meat particles. The resulting suspension was centrifuged at 2,000 R.C.F. for 30 minutes and the supernate was discarded. The bacterial deposits were resuspended in 0.85% NaCl and washed 6 times. After the last centrifugation, the pellet was resuspended in 3 volumes of 0.85% NaCl. The final suspension of C. parvum was then heat-killed by incubation in a 70°C water bath for 60 minutes. The suspension was then finally checked for sterility and if the suspension was

not immediately used, enough formalin was added to make a final concentration of 1%. The dry weight of the suspension was then determined by heating 1.00 ml of the suspension at 100°C in a tared flask until constant weight was established.

Protein Nitrogen Analysis by the Micro-Kjeldahl Method

Introductory note

Protein estimation procedures are often standardized by the micro-Kjeldahl nitrogen analysis because of the precision and accuracy of this method. The procedure is divided into three stages: the oxidation of the protein, the distillation of ammonia and the acid titration. The protein is oxidized by boiling in concentrated sulphuric acid in the presence of K_2SO_4, which raises the boiling point, and catalysts such as Cu, Se, or Hg. The carbon and hydrogen constituents of the protein are converted to CO_2 and H_2O respectively and the protein nitrogen is transformed to ammonia, which in the presence of H_2SO_4 becomes $(NH_4)_2SO_4$. The most important aspect of this oxidation step is that the temperature is high enough to ensure complete decomposition of the protein but low enough so that there is no loss of ammonia; a suitable temperature range of 350 - 400°C is recommended (Kabat and Mayer, 1961). The distillation step is carried out in a Markham Still. Concentrated NaOH liberates ammonia which is then driven out of solution by steam distillation and the distillate is collected in a vessel containing boric acid.

The ammonia is thus trapped in solution as ammonium borate. The number of moles of ammonia, hence nitrogen, may easily be determined by titration of the solution with standard HCl or H_2SO_4. Methyl red is a suitable indicator because boric acid does not turn the indicator red whereas slight excess of a strong acid elicits the colour change. The following method is designed to detect from 20 - 200 ug N. Estimation of the dry weight of protein may be performed by multiplying the weight of nitrogen by 6.25; this will only give an estimation due to the variation of total nitrogen content from protein to protein.

Materials

1. Concentrated sulphuric acid.
2. 2% Boric acid.
3. 40% Sodium hydroxide.
4. 0.01 N sulphuric or hydrochloric acid.
5. Catalyst mixture:

K_2SO_4	80 g
$CuSO_4$	20 g
Selenium	100 mg

The mixture was ground to a fine powder in a mortar and pestle.

6. Titration indicator, Kabat and Mayer (1961):

1% Methylene blue in distilled water;

Saturated solution of methyl red in 95% ethyl alcohol.

The indicator was prepared by mixing 1.5 ml of the

methylene blue solution with 12.5 ml of the methyl red solution.

Procedure

Six test tube digestion flasks were used in every nitrogen determination. The desired amount of the unknown protein (Exp.) solution was volumetrically pipetted into three digestion flasks and the volume made up to 0.5 ml with distilled water. The fourth digestion flask contained 0.50 ml of a protein standard and the last two digestion flasks served as controls (Con.) and contained 0.5 ml of distilled water. One spatula of the catalyst mixture (approximately 50 mg) was placed into all of the digestion flasks followed by 0.8 ml of concentrated sulphuric acid. The tubes were heated over glowing electric coils for 2 1/2 hours. After cooling, the contents of each flask were quantitatively transferred to the Markham Still and 5.0 ml of 40% NaOH were added and the distillate collected in a vessel containing 3 ml of 2% boric acid until 20 ml had distilled over. Four drops of the titration indicator were added to all six vessels and titration was carried out with 0.01 N H_2SO_4 until the first trace of purple colour remained for 30 seconds. The control titrations were averaged and subtracted from the average of the experimental titration. The calculation of ug N/1.00 ml protein solution was performed as follows:

(ml H_2SO_4, Exp. - ml H_2SO_4, Con.) x 140 x 2 = ug N/1.00 ml

Estimation of Protein by the Folin-Ciocalteu Method

Explanatory note

This is a colorimetric method for the rapid estimation of small quantities of protein, 50 - 500 ug. The colour developed by the Folin-Ciocalteu phenol reagent in basic conditions is due predominantly to reaction of the Folin reagent with the tyrosine and tryptophane residues of the protein. The colour is also dependent upon the presence of SH and other reducing groups and the length of time the protein is exposed to the sodium hydroxide before the addition of the Folin reagent (Kabat and Mayer, 1961). The concentration of protein is directly proportional to the optical density of the coloured solution after completion of the reaction. The method used is modified from the one described by Lowry, Rosebrough, Farr and Randall (1951).

Materials

1. Solution A — 2% Na_2CO_3 in 0.10 N NaOH
2. Solution B — 1% $CuSO_4$ and 2% sodium tartrate, mixed 1:1 before using.
3. Solution C — 50 volumes of solution A mixed with 1 volume of solution B immediately before using.
4. Folin reagent — This preparation was purchased from British Drug Houses, Ltd., Poole, England, and was 2 N in acidity. The Folin reagent was diluted 1:2 with dionized water immediately before using.

Procedure

All unknown protein solutions were run in triplicate along with triplicate protein standards and controls. To a series of tubes 1.00 ml of the unknown protein, standard or distilled water was added. 4.5 ml of solution C were then added to each tube and the tubes were immediately mixed and allowed to stand at room temperature for 10 minutes. 0.50 ml of the 1:2 Folin reagent was then added, mixed and left at room temperature for 30 minutes. The optical densities of the various tubes were read against the control tubes at 540 mu (Unicam S.P. 1300 with Ilford filter # 625); alternatively greater sensitivity was achieved by reading the optical densities at 750 mu in a Beckman or Unicam spectrophotometer. The protein concentration was determined by comparing the unknown with the standard protein solution; standard curves were not used as it was felt that this would result in decreased accuracy.

Preparation of Bovine Serum Albumin Antigens

Explanatory note

During the preparation of the various bovine serum albumin antigens great care was taken to avoid any microbial contamination. The stock BSA solutions were frozen at -20°C and thawed only once and the BSA which was was not used was discarded. When preparing the stock BSA solution frothing was carefully avoided as this encourages denaturation of the BSA molecule.

Materials

1. Bovine serum albumin (Cohn Fraction V) was purchased from Armour Pharmaceutical.
2. Alhydrogel (1.3% Al_2O_3) was a generous gift from the Superfos Export Co., Copenhagen, Denmark.
3. 0.85% NaCl

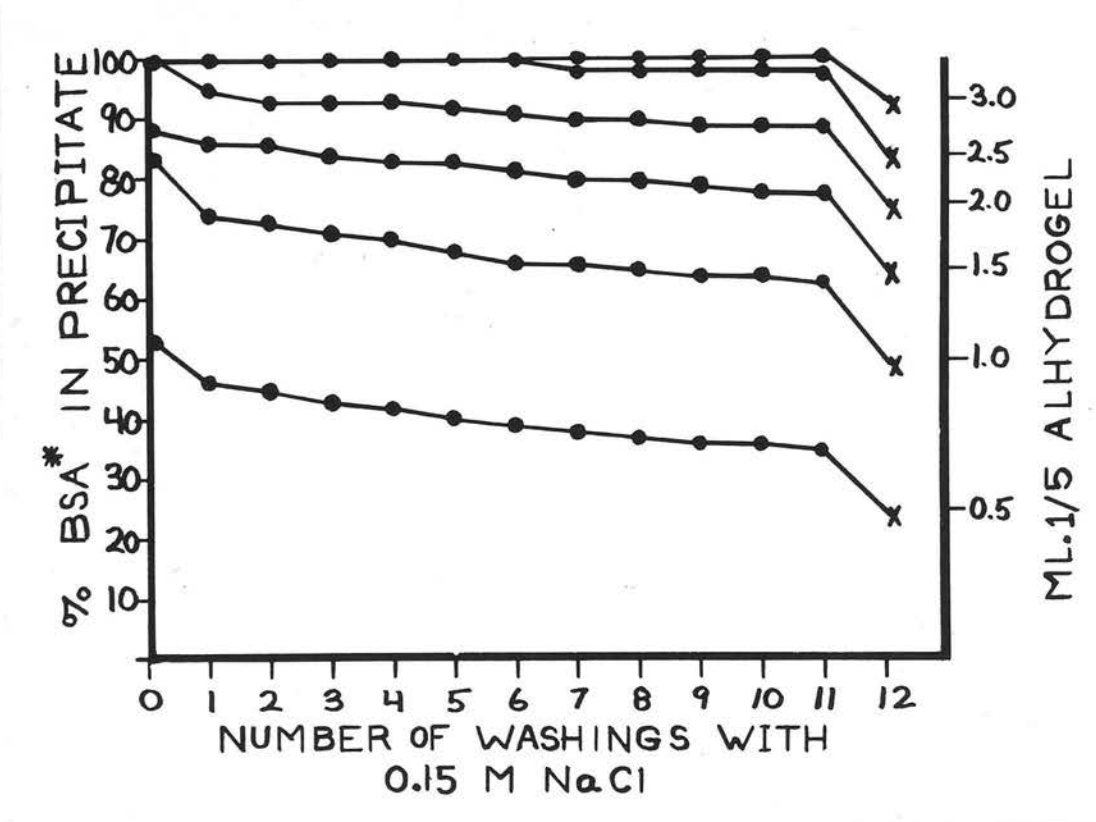
Procedure

Native bovine albumin (NBA): A 100 mg/ml stock solution of NBA was prepared by carefully dissolving 10 g of bovine serum albumin in 70 ml of 0.85% NaCl and bringing the final volume to 100 ml. The NBA solution was then sterilized by Seitz-filtration and stored at -20°C.

Centrifuged bovine albumin (CBA): Four ml quantities of NBA were centrifuged at 40,000 R.P.M. for 60 minutes (8.2×10^6 g-min) in an MSE Superspeed 40, 3 x 5 ml swing-out rotor. At the end of the 60 minute centrifugation period the rotor was allowed to come down without braking and the top 2 ml of solution for each tube was carefully drawn off with a 2 ml volumetric pipette; the CBA solution was then immediately used. It was found on three separate occasions, using the Folin-Ciocalteu method for protein estimation, that the top 2 ml of each tube contained 45 mg/ml and the bottom 2 ml of each tube contained 55 mg/ml. Therefore, because of this previous standardization of protein distribution, exact amounts of CBA could be employed immediately after centrifugation thus minimizing any protein denaturation that might have occurred with time.

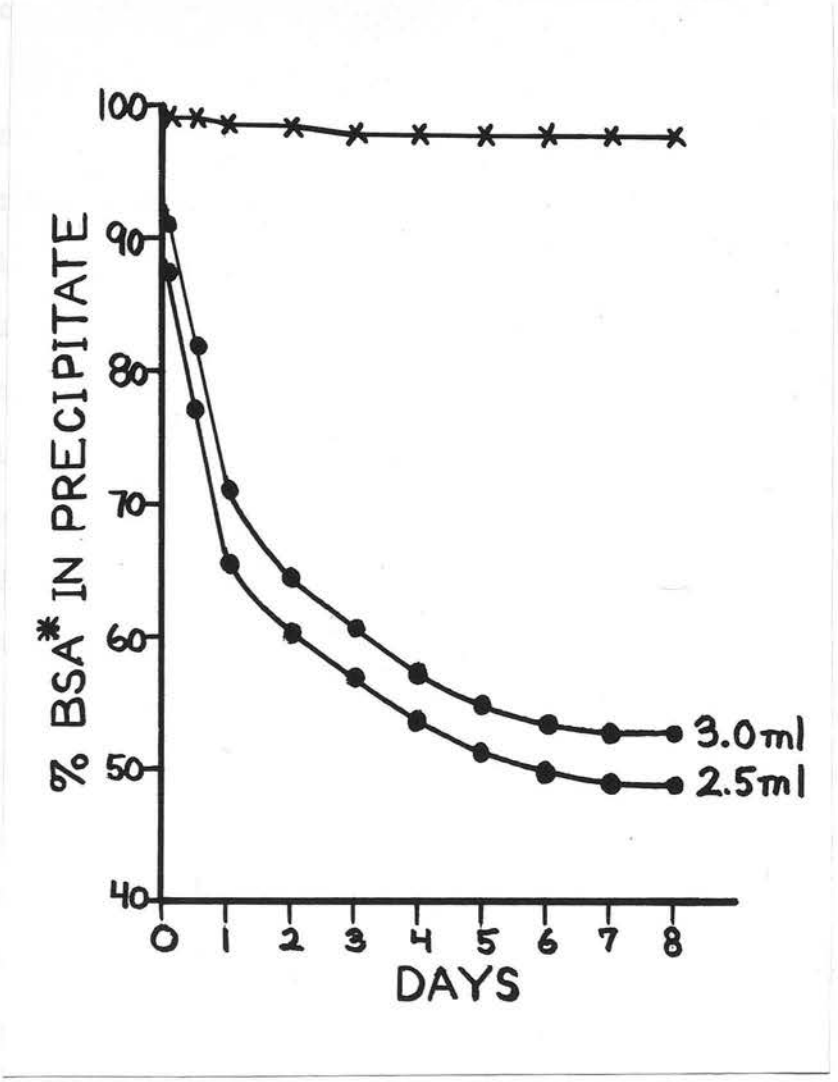
Alum-precipitated centrifuged bovine albumin (ACBA): The ACBA was prepared as an aluminium hydroxide precipitate by adding, with mixing, 50 mg of CBA to a mixture of 6 ml of Alhydrogel and 6 ml of sterile 0.85% NaCl. The mixture was allowed to equilibrate at room temperature for 60 minutes and then overnight at $4^{o}C$. The precipitate was centrifuged at 1,000 R.C.F. for 10 minutes and the supernate discarded. The precipitate was then resuspended in 2 - 3 ml of sterile 0.85% NaCl and passed through a 19 gauge needle 3 to 4 times in order to disperse any large aggregates. The optimal ratio of alhydrogel/mg CBA was determined by adding constant amounts of trace-labelled I^{131}-BSA to varying amounts of a 1/5 dilution of alhydrogel. The precipitates were allowed to form and were washed extensively with 0.15 M NaCl. It was found that 2.5 ml of a 1/5 alhydrogel preparation completely precipitated 5 mg of BSA (see Fig. 2.1). The stability of the Al_2O_3-BSA complexes was further checked by incubating the ACBA in 1/10 normal rabbit serum and washing extensively with the same media. It can be seen in Figure 2.2 that by increasing the quantity of alhydrogel to 3.0 ml a slightly more stable complex was formed; even after one week of extensive washing with 1/10 normal rabbit serum greater than 50% of the BSA remained bound to the Al_2O_3.

Figure 2.1



Percent 5 mg BSA* precipitated by varying quantities of 1/5 Alhydrogel and remaining after successive washings with 0.15 M NaCl (●) or 1/10 normal rabbit serum (X).

Figure 2.2



Percent 5 mg BSA* precipitated by 2.5 ml or 3.0 ml of 1/5 Alhydrogel and remaining after successive washings with 1/10 normal rabbit serum (●) or with 0.15 M NaCl (X).

Preparation of Trace-labelled Iodine^{131}-Bovine Serum Albumin

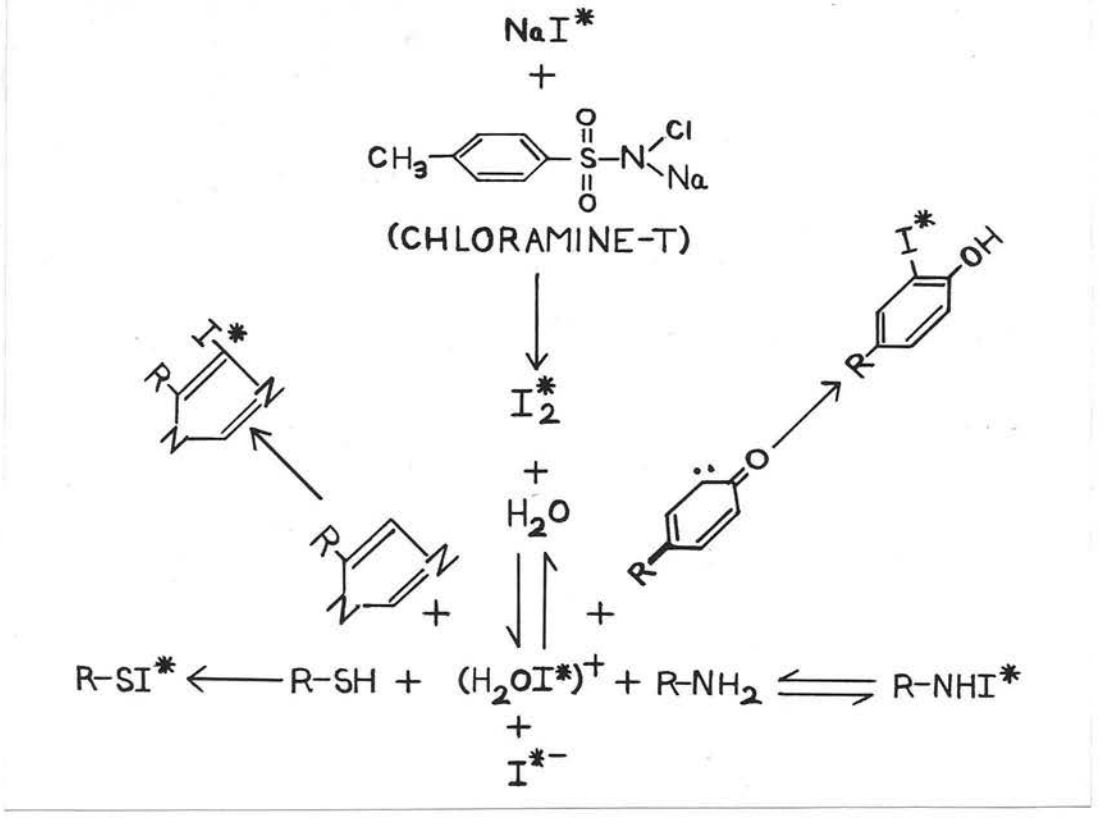
Explanatory note

There are two important points concerning the iodination of a protein: 1) one atom or less of iodine per molecule of protein should be the substitution ratio as this has been shown to result in a protein molecule immunochemically identical with the native protein molecule and 2) the method of iodination should be of the highest efficiency of incorporation thus decreasing the hazards of working with large quantities of the isotope. A slight modification of the Chloramine T method for iodination described by Hunter and Greenwood (1962) was used. Reference should be made to Figure 2.3 which shows the probable reactions which take place during the iodination process (Kabat and Mayer, 1961). NaI^{131} is oxidized by Chloramine T to free iodine which then reacts with water forming H_2OI^+ which in turn reacts predominantly under alkaline conditions, with the phenolate ion in the tyrosine residue. Although iodination proceeds almost exclusively to the tyrosine residue, it can be seen that other groups on the protein molecule may react with H_2OI^+ complex. The following method for iodination of BSA is greater than 90% efficient.

Materials

1. Borate buffer (see Farr technique).
2. Carrier-free NaI^{131} free of reducing agent: 2 - 4 mc (purchased from Radiochemical Centre, Amersham, Bucks.).

Figure 2.3



Probable reactions occuring during the iodination of protein with NaI^{131} using the Chloramine-T method; R denotes the peptide chain of the protein molecule.

3. KI

4. Chloramine T solution (sodium p-toluenesulphonchloramide):
   50 mg Chloramine T/100 ml distilled water.

5. BSA solution in borate buffer: 10 mg BSA/1.00 ml.

Procedure

In a small reaction flask containing 2 - 4 mc carrier-free iodine^{131}, 0.2 ml of borate buffer pH 8.4 was added. 5 mg of BSA in 0.5 ml of borate buffer was then added with rapid stirring followed immediately by 100 ug of Chloramine T in 0.2 ml of distilled water. The solution was allowed to react for 60 seconds and then 4 ml of borate buffer was added. The solution was dialysed against borate buffer for 24 hours and then against numerous changes of borate buffer containing a few crystals of KI carrier for 2 - 3 additional days or until the radioactivity of the dialysate remained constant for a 24 hour period. The concentration of I^{131}-BSA in ug N/1.00 ml was determined by the micro-Kjeldahl method. The I^{131}-BSA preparations were used only if greater than 99% of the radioactivity was precipitable by 10% trichloroacetic acid. The I^{131}-BSA was stored in small aliquots at -20°C and thawed only once, the unused material being discarded.

Standardization and Counting Procedures for Iodine^{131}

Explanatory note

The procedure for counting gamma ray emission is in

itself simple and is based on the following principles. A gamma ray scintillation spectrometer is basically composed of a NaI crystal, photomultiplier, amplifier and pulse height analyzer. The gamma ray passing through the NaI crystal causes an excitation of the crystalline matrix bringing about the release of photons which are picked up by the photomultiplier; this amplifies the signal which is then taken up by the amplifier where further amplification is performed. The pulse height analyzer discriminates between different pulse heights which are generated by gamma rays of different quantum energies, or in counting terminology sets the "window" for counting. Iodine^{131} has 5 quantum energy levels of gamma and beta radiation. The emission which is counted is the gamma ray with the quantum energy of 0.364 MeV. The counting efficiency of most gamma scintillation spectrometers is between 30 - 40%; the counting efficiency is the number of counts per number of disintegrations times 100. There are three important factors which must be known and controlled during counting: 1) the access time of the counting circuits (i.e., the "dead time" of the counting equipment), 2) the influence of geometry upon counting and 3) the exact window setting must be determined so that temperature fluctuations and/or voltage changes will not increase or decrease the counting efficiency of the apparatus.

Counting procedure

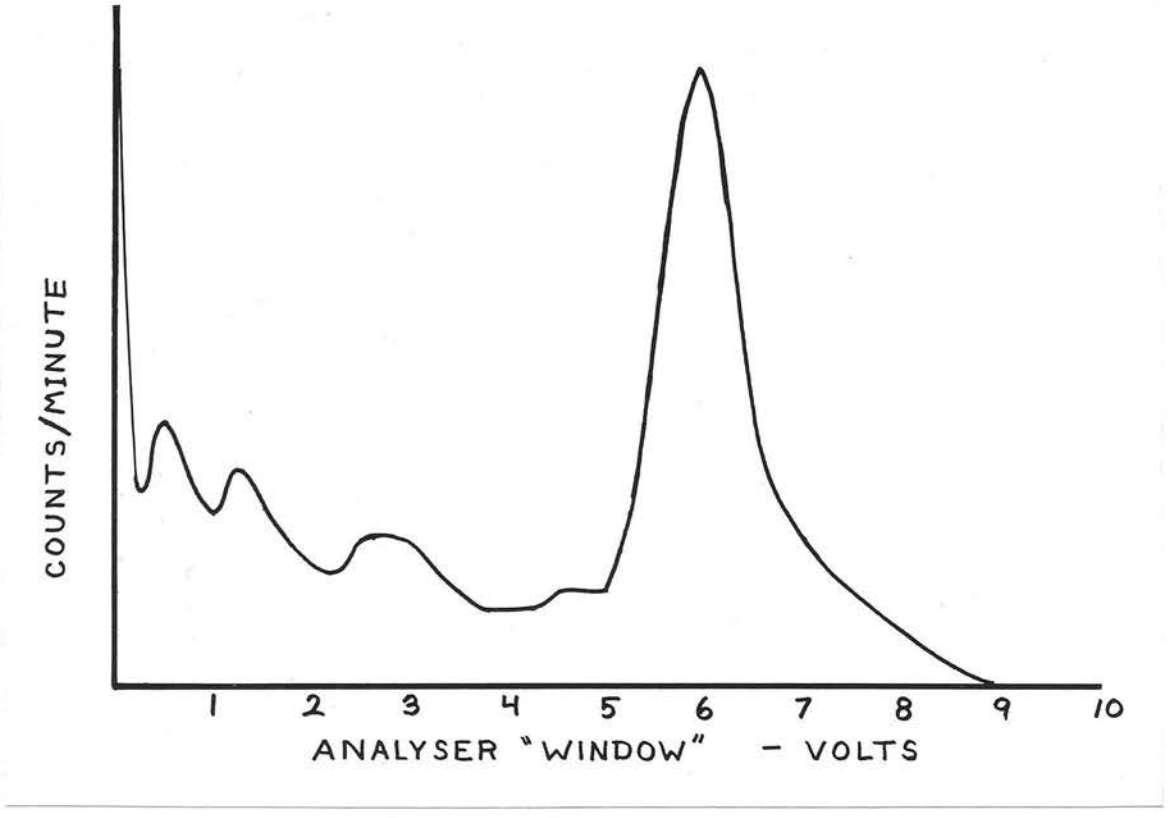
A Nuclear Enterprises "Gamma-Matic" Mark 1-A Scintillation

Spectrometer was used throughout this study. The equipment had a 2 inch, well-type NaI crystal. Although the exact dead time of this counting equipment was not known, it was determined that at least 5×10^5 counts/minute did not exceed the dead time of the counting circuits; not more than 1.5×10^5 counts/minute were used in any of the experiments in this study. The geometry of the radioactive source did influence the counting spectrum of Iodine^{131}; it was found that a point source of Iodine^{131} resulted in a broader spectrum than did a source with expanded geometry. As the engineers at Nuclear Enterprises could not explain this phenomenon, which is the opposite from the expected, the author did not carry out any further investigation. The geometry in all experimental procedures, however, was constant being a precipitate in the bottom of a Wasserman tube. The following settings were used to set the window on the Mark 1-A and Figure 2.4 shows the final calibration using the Analyzer to set pulse height. The best window to maintain stability of efficiency is E, equal to 4.5 volts and E, equal to 5.0 volts.

Output voltage		1,000 volts
Amplifier, gain	Coarse	250 volts
	Fine	0.5 volts
Analyzer	E	4.5 volts
	Δ E	5.0 volts

Analysis of the counting error is a relatively straightforward procedure. The probable counting error is equal to the square root of the total number of counts divided by the

Figure 2.4



Calibration of the analyser "window" for the Mark 1-A, Nuclear Enterprises "Gamma-Matic" Scintillation Spectrometer; window setting from 4.5 - 9.5 volts.

total number of counts times 100. The maximum counting error is three times the probable counting error. Thus if there are 10,000 counts, then the probable counting error is 1% and the maximum counting error is 3%.

The Ammonium Sulphate Method for Determining the Antigen-binding Capacity (The Farr Technique)

Explanatory note

The Farr technique is one of the few qualitative and quantitative tests which measure the primary interaction between antigen and antibody. The technique is based upon the following well tested and controlled observations: 1) Iodine^{131} labelled bovine serum albumin (BSA*) is soluble in 50% saturated ammonium sulphate, 2) BSA* which is specifically bound to antibody in the zone of antigen excess is insoluble in 50% saturated ammonium sulphate and 3) following precipitation with 50% saturated ammonium sulphate, antibody does not release, exchange or bind additional BSA*. Because of the third observation the amount of BSA* in the precipitate is a close approximation of the amount of BSA* bound at equilibrium in solution. Not only does the Farr technique determine the extent to which an antiserum can bind antigen (i.e., it reflects the quantity of antibody) but by determining the antigen-binding capacity (ABC) at two different antigen concentrations, the effect of dilution per cent can be determined

which is proportional to the relative binding affinity of the antibody molecules (i.e., the quality of the antibody). The Farr technique is the only immunochemical procedure available at present which can fully characterize the quantity and quality of an antiserum to a protein antigen.

The procedure for determining the ABC of an antiserum is straightforward. Serial dilutions of the antiserum starting at 1/10 are made in duplicate, and constant amounts of BSA^{*} are added to each tube. After incubating at $4^{o}C$ for 18 hours an equal volume of saturated ammonium sulphate is added and the resulting precipitate is centrifuged and washed with 50% saturated ammonium sulphate. After counting the precipitates and correcting for non-specific activity, the percentage of BSA^{*} bound at each antibody dilution is determined. By plotting the percentage of BSA^{*} bound against the $\log_{10}$ reciprocal antibody dilution, the theoretical dilution of antibody which would bind 33% of BSA^{*} is determined. The ABC values are then expressed as ug N BSA^{*} bound/ml of undiluted antiserum. The following procedure is based upon the method described by Farr (1958) and Minden and Farr (1967).

Materials

1. Borate buffer (pH 8.3 - 8.5, /2 0.1):

   6.184 g boric acid

   9.536 g borax (sodium tetraborate)

   4.384 g NaCl

   Made up to one litre with distilled water.

2. Saturated ammonium sulphate (SAS) at 4°C, sp. gr. 1.240
3. Half saturated ammonium sulphate (SAS/2).
   One volume of SAS was added to one volume of borate buffer.
4. 1/10 normal sera.
   One volume of normal sera was added to 9 volumes of borate buffer.
5. 1/100 normal sera.
   One volume of normal sera was added to 99 volumes of borate buffer.
6. Trace-labelled Iodine^{131}-bovine serum albumin.
   The stock solution of BSA* was diluted with 1/100 normal serum to give two solutions of 0.40 ug N BSA*/ml and 0.040 ug N BSA*/ml.

Procedure

All of the dilutions and transfer steps were done with volumetric, blow-out pipettes unless otherwise stated. All of the antisera were initially diluted 1/10 by adding 0.40 ml of antiserum to 3.60 ml of borate buffer. Thereafter either 1/3, 1/4 or 1/5 serial dilutions were made employing 1/10 normal sera as diluent to keep the protein concentration constant in all the tubes. Usually 1/3 serial dilutions were made in the early primary response antisera, 1/4 dilutions in the late primary response antisera and 1/5 throughout the secondary response antisera. Starting with the highest (most dilute) antiserum dilution 0.50 ml amounts were transferred to four suitably labelled Wasserman tubes (duplicate tubes for

the 0.20 ug N BSA* test and duplicate tubes for the 0.020 ug N BSA* test). Using the same pipette the next highest dilution was similarly transferred to four Wasserman tubes and so on until the initial 1/10 dilution was transferred. 0.50 ml volumes of 1/10 normal sera were added to 16 control tubes (8 for each BSA* concentration). Using a 1 ml automatic syringe with a valve and canula attachment (R.B. Turner and Co. Ltd., London), 0.50 ml amounts of 0.40 ug N BSA*/ml solution or 0.040 ml ug N BSA*/ml solution were added to experimental and control tubes. The tubes were then incubated at 4°C overnight (approximately 18 hours). The tubes were immersed in an ice-water bath equilibrated to 4°C and 1.0 ml of SAS was added with an automatic syringe to each tube which was then immediately mixed with the aid of a vibrating mixer. 1.0 ml quantities of SAS were added to 4 control tubes containing the highest concentration of BSA* and to 4 control tubes containing the lowest concentration of BSA*. Similarly 1.0 ml amounts of 20% trichloroacetic acid (TCA) were added to the remaining 8 control tubes. The tubes were allowed to incubate at 4°C for 30 minutes and then were centrifuged in an MSE "Major" refrigerated (4°C) centrifuge at 2,000 R.P.M. (1,500 R.C.F.) for 30 minutes. The supernates were poured off and the tubes were immersed in a 4°C ice-water bath and 3 ml of SAS/2 were added to each tube, excluding the TCA control tubes, with the aid of a 5 ml automatic syringe. The contents of the tubes were mixed with the vibrating mixer and incubated for 30 minutes at 4°C and then centrifuged (at 4°C) for 30 minutes at 2,000 R.P.M. and the

supernates poured off. All of the tubes were then counted in a Nuclear Enterprises "Gamma-Matic" scintillation spectrometer Mark 1-A with a 2 inch well-type NaI crystal. The counting period was adjusted so that the probable counting error of every experimental tube above 20% BSA^{*} bound did not exceed 2%.

One of two methods for calculating the percentage of BSA^{*} specifically bound was used.

Method A: The duplicate experimental tubes (EXP) were averaged and background activity was subtracted. The control tubes which were precipitated with 10% TCA (CONT A) and the control tubes treated with SAS (CONT B) were averaged and background activity was subtracted. CONT A was used to give the number of counts representing 100% precipitation of BSA^{*}; CONT B gives the number of counts which are non-specifically precipitated. In order to correct the experimental tube counts for non-specific activity a supernate correction factor (SCF) must be determined. The SCF is based upon the observation that the amount of non-specific activity is directly proportional to the amount of BSA^{*} which remained in the supernate after SAS/2 precipitation; the SCF is calculated CONT B/(CONT A-CONT B). The calculation of the specific percentage binding was calculated as follows:

$$\%\ BSA^{*}\ \text{bound} = (EXP - ((CONT\ A - EXP)SCF))/CONT\ A \times 100$$

Method B: This procedure is mathematically the same as Method A but is a more rapid means of calculating the percentage binding. The difference is that a precipitate correction factor (PCF) is used in place of the SCF. The PCF

is calculated as 1/(CONT A - CONT B). The calculation of the specific percentage binding was calculated as follows:

$$\% \text{ BSA}^{*} \text{ bound} = 100 - (\text{CONT A} - \text{EXP})\text{PCF}$$

The data is graphed on a semi-log plot, the percentage binding being on the linear y-axis and the x-axis being the $\log_{10}$ reciprocal antiserum dilution. The 33% endpoint was calculated by drawing a straight line through any points where the percentage binding was less than 90% but greater than 30%. The theoretical antiserum dilution which would bind 33% of the antigen added was then read directly from the plot. The antigen-binding capacity was then calculated as follows:

$$\text{ABC-33} = \text{Reciprocal end-point dilution} \times \text{ug N BSA}^{*} \text{ added} \times .33 \times 2$$

The effect of dilution percentage was calculated as:

$$(\text{ABC-33}(0.020 \text{ ug N BSA}^{*})/\text{ABC-33}(0.20 \text{ ug N BSA}^{*}) \times 100$$

RESULTS

Experiment 1

The Effects of the Physical State of the Antigen and of *Corynebacterium parvum* on the Quantity and Quality of the Antibodies produced to Intravenous Injection of Bovine Serum Albumin in Rabbits.

The objectives of the first experiment were to characterize the humoral antibody response to aggregate-free (centrifuged) bovine albumin (CBA), particulate (alum-precipitated) centrifuged bovine albumin (ACBA) and partially aggregated native bovine albumin (NBA); secondly, the adjuvant properties of *C. parvum* were studied using CBA, ACBA and NBA as antigens. The Farr technique was employed in this and the following experiments to assess both the quantity and quality (i.e., change in the relative binding affinity) of the antibodies produced: 0.20 ug N BSA* and 0.020 ug N BSA* were simultaneously used to determine two antigen-binding capacities of each antiserum. The design of the experiment is shown in Table 3.1.

Primary response

Three month old New Zealand White rabbits of both sexes were employed. Six parallel groups were run, three groups of rabbits received intravenous injections of 50 mg CBA, NBA, or ACBA on day 0. The three remaining groups were injected intravenously with 10 mg of a heat-killed suspension of *C. parvum* 6 days prior to the intravenous injections of

Table 3.1

Design of Experiment 1

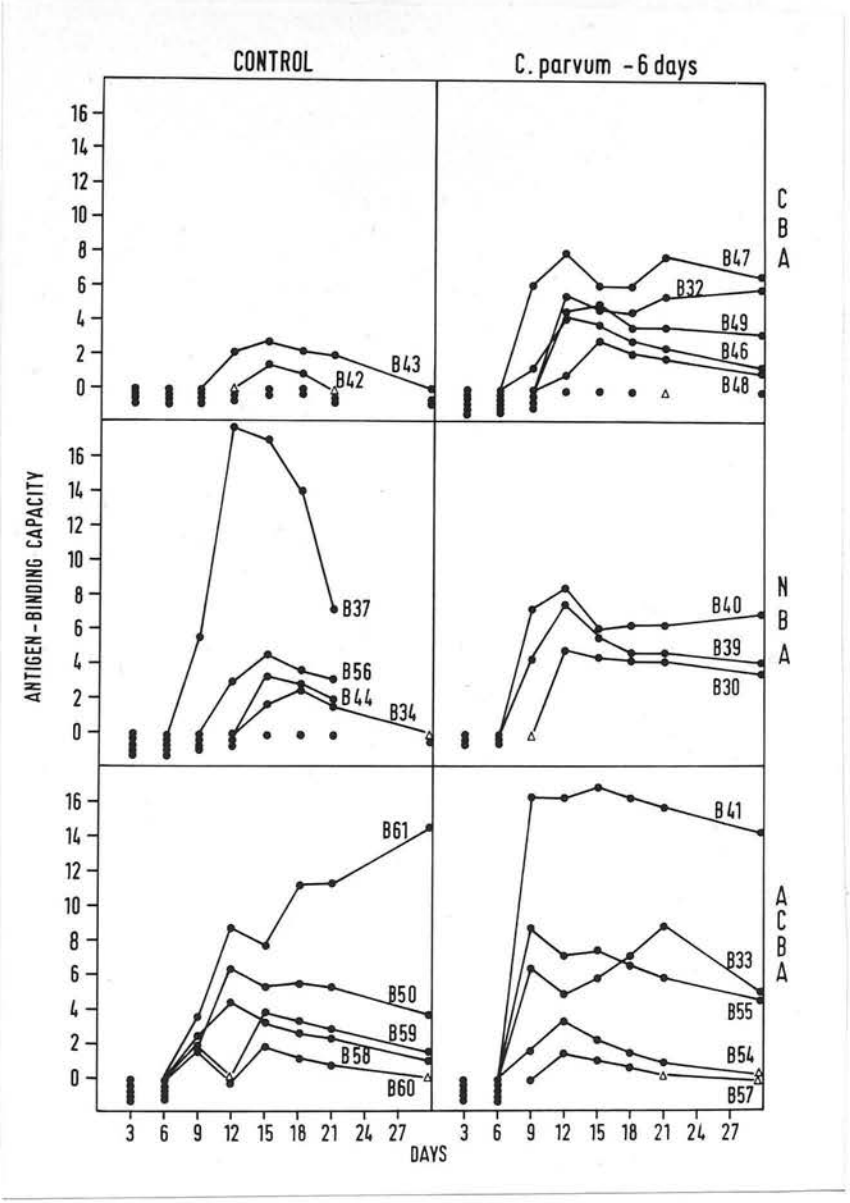
#	Sex	Wt.(Kg)	Pretreatment Day - 6	Primary Day 0	Secondary Day 30
B30	M	3.0	10 mg *C. parvum* I.V.	50 mg NBA I.V.	50 mg NBA I.V.
B39	F	3.0			
B40	F	2.3			
B34	M	2.5	None	50 mg NBA I.V.	50 mg NBA I.V.
B35	F	2.7			
B37	F	2.5			
B44	F	2.2			
B56	M	2.6			
B32	M	2.5	10 mg *C. parvum* I.V.	50 mg CBA I.V.	50 mg CBA I.V.
B38	M	2.5			
B46	M	3.0			
B47	F	2.7			
B48	F	2.7			
B49	F	2.5			
B42	M	2.7	None	50 mg CBA I.V.	50 mg CBA I.V.
B43	F	2.3			
B45	F	2.5			
B53	F	2.5			
B33	M	3.0	10 mg *C. parvum* I.V.	50 mg ACBA I.V.	50 mg ACBA I.V.
B41	F	2.5			
B54	M	3.0			
B55	M	2.7			
B57	F	2.5			
B58	M	3.0	None	50 mg ACBA I.V.	50 mg ACBA I.V.
B59	F	2.7			
B60	F	2.5			
B61	F	2.5			
B50	M	2.5			

50 mg CBA, NBA or ACBA. The results of the primary responses are shown in Figure 3.1 which reflects the levels of anti-BSA antibodies produced and in Figure 3.2 which shows the increase of the relative binding affinity of the antibodies. More detailed information is given in Table 3.2.

Antigen-binding capacity: The results expressed in Figure 3.1 show that in general those rabbits in the control group receiving ACBA responded to a greater degree than either the CBA or NBA injected control groups. In contrast all three C. parvum injected groups gave elevated ABC values. It is noteworthy that there is a marked difference between the control CBA and C. parvum-CBA groups both in ABC values and the number of rabbits responding in each group. This situation is similar in the control NBA and C. parvum-NBA groups with the exception of rabbit B 37. There was, however, no significant difference between the control ACBA and C. parvum-ACBA groups. It should also be noted that, in the control ACBA and C. parvum treated groups, a large number of rabbits elicited a "biphasic" and sustained immune response with respect to ABC magnitude.

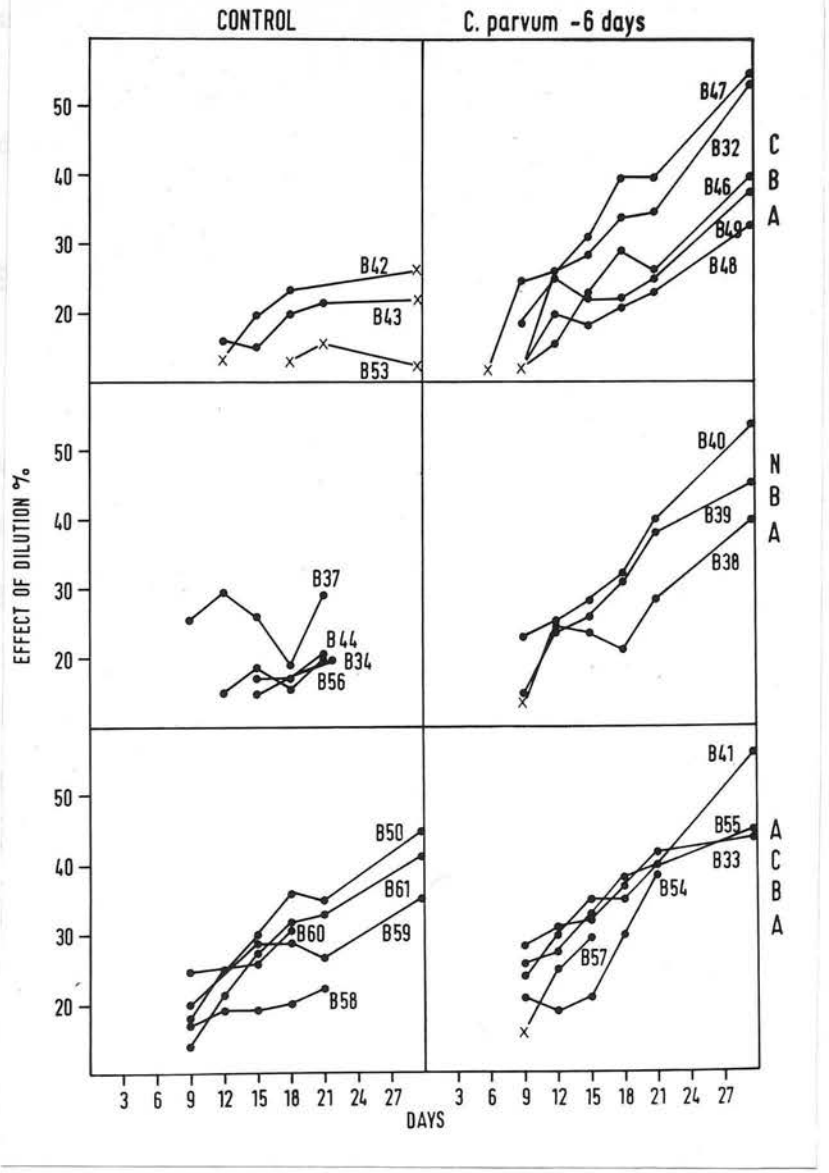
Effect of dilution: Figure 3.2 demonstrates the effect that dilution of the BSA* antigen has upon the ABC values which, as stated previously, gives an indication of the relative binding affinity of an antiserum; this enabled qualitative comparisons to be made between the antisera produced by the various groups. It is apparent that not only are the effects of dilution percentage values greater in the

Figure 3.1



Primary response: Antigen-binding capacity (Reciprocal ABC-33 end point x 0.20 ug N BSA x 2 x 0.33) of control and Corynebacterium parvum (-6 days) treated groups given intravenous injections of centrifuged bovine albumin (CBA), native bovine albumin (NBA) or alum-precipitated centrifuged bovine albumin (ACBA) on day 0. Δ, insufficient antibody to give a positive ABC at a 1:10 dilution of antiserum.

Figure 3.2



Primary response: Effect of dilution % ((ABC-33, 0.02 ug N BSA/ABC-33, 0.20 ug N BSA) x 100) of control and Corynebacterium parvum (-6 days) treated groups given intravenous injections of CBA, NBA or ACBA on day 0. X indicates the estimated effect of dilution % from the % binding of a 1:10 dilution of antiserum.

control ACBA and C. parvum treated groups, but the rates of increase are more rapid. This indicates that not only is there more antibody produced in the control ACBA and C. parvum treated groups, as seen in Figure 3.1, but the antibodies produced have a greater affinity for the BSA molecule.

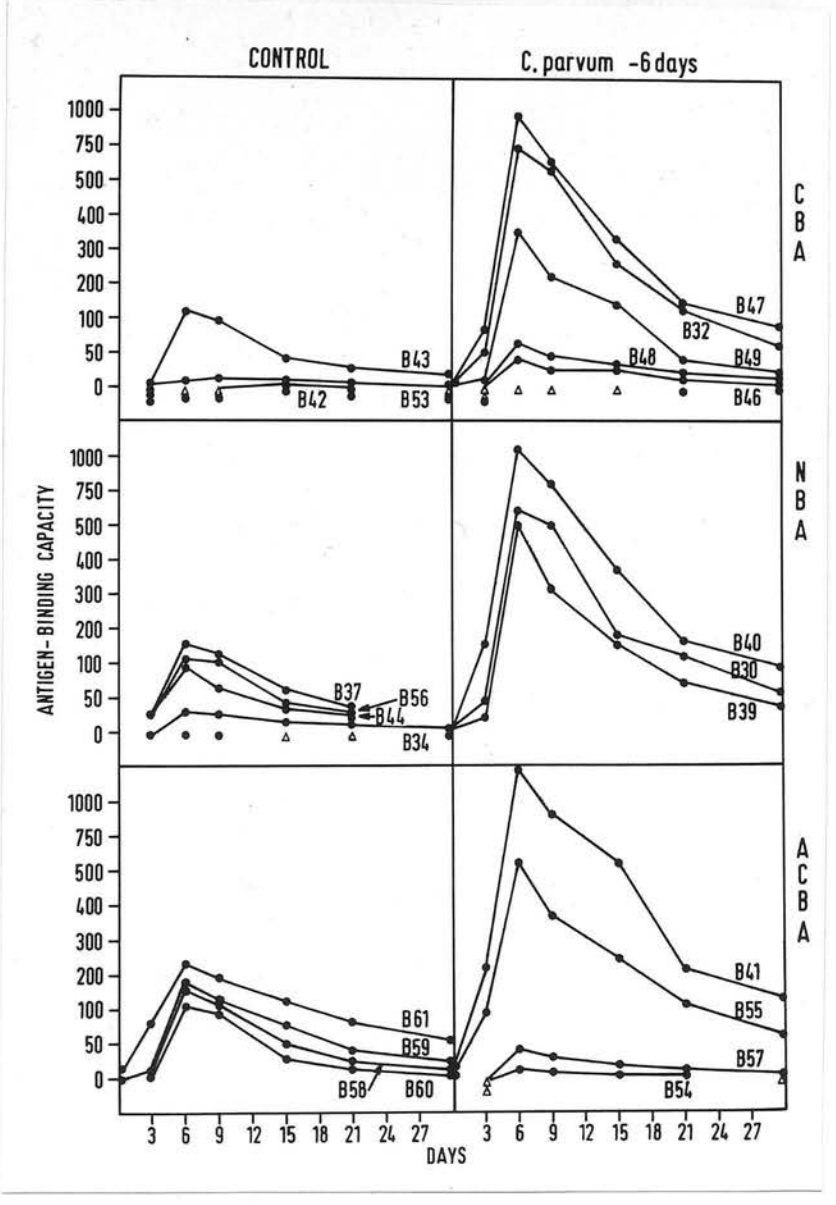
Secondary response

On day 30 of the primary response, all of the surviving rabbits were given intravenous injections of 50 mg of the bovine antigen in the same physical form as they received for the primary stimulus. None of the rabbits were injected with C. parvum. The results of the secondary response are shown in Figure 3.3 which reflects the levels of anti-BSA antibody produced and in Figure 3.4 which shows the increase of the relative binding affinity of the antibodies; more detailed information is given in Table 3.3.

Antigen-binding capacity: The results of the quantitative production of anti-BSA antibodies are seen in Figure 3.3. It is evident that a greater number of rabbits receiving C. parvum prior to the primary stimulation with the respective bovine albumin antigens, irrespective of their physical state, become hyperreactive (in comparison to the control groups) shortly after secondary exposure to the antigen. The most profound difference is seen between the control CBA and C. parvum-CBA groups.

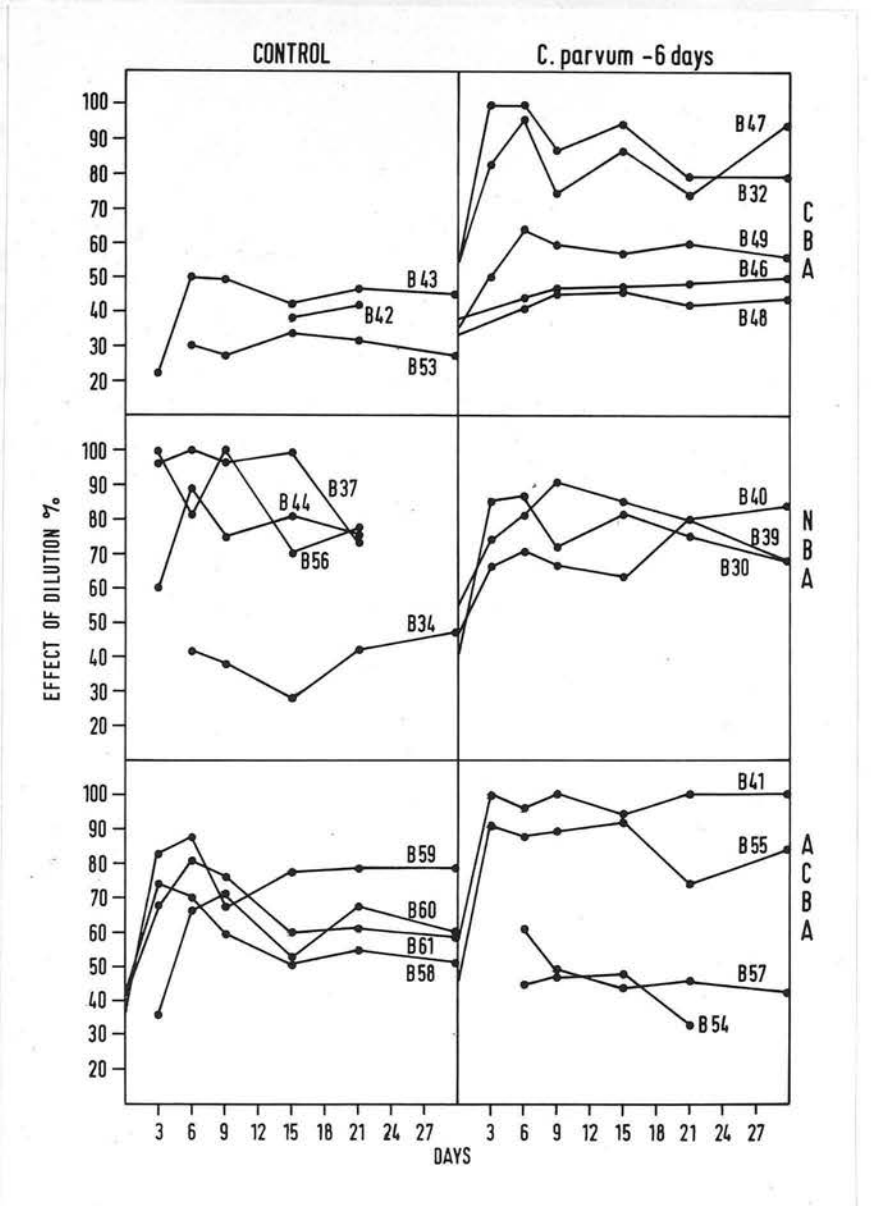
Effect of dilution: In all of the groups there is in general an immediate, rapid increase in the effect of dilution

Figure 3.3



Secondary response: Antigen-binding capacity (Reciprocal ABC-33 end point x 0.20 ug N BSA x 2 x 0.33) of control and Corynebacterium parvum (-6 days) treated groups given secondary intravenous injections of CBA, NBA and ACBA on day 30 of the primary response (i.e., day 0 of the secondary response). Δ, insufficient antibody to give a positive ABC at a 1:10 dilution of antiserum.

Figure 3.4



Secondary response: Effect of dilution % ((ABC-33, 0.02 ug N BSA/ABC-33, 0.20 ug N BSA) x 100) of control and Corynebacterium parvum (-6 days) treated groups given secondary intravenous injections of CBA, NBA or ACBA on day 30 of the primary response (i.e., day 0 of the secondary response).

percentage as seen in Figure 3.4. It is again noteworthy that in the C. parvum-CBA group there is a significant difference in the magnitude of the relative binding affinity of the antibody as compared to the control CBA group.

Conclusion

The physical state of bovine albumin profoundly influences the development of the humoral antibody response. CBA induced a hyporesponsive state to BSA characterized by suppressed ABC values and a decreased evolution of the relative binding affinity of any antibodies which were produced. ACBA on the other hand induced a hyperreactive state characterized by elevated ABC values and a rapid increase in the relative binding affinity of the antibodies. NBA initiated an immune response intermediate between CBA and ACBA. It was found that C. parvum when injected 6 days prior to the injection of CBA, NBA or ACBA induced a hyperreactive state to BSA, irrespective of the physical state of the bovine antigen.

Table 3.2: Primary Response Data of Experiment 1

	Day 6			Day 9			Day 12			Day 15		
	.02	.20	%	.02	.20	%	.02	.20	%	.02	.20	%
B30	-	-		* 9.6	* 7.0		1.17	4.75	24.6	1.04	4.42	23.5
B39	-	-		.62	4.22	14.5	1.76	7.39	23.8	1.48	5.64	26.2
B40	-	-		1.66	7.13	23.3	1.98	8.32	23.8	1.66	5.94	27.9
B34	-	-		-	-		* 4.6	* 4.4		.29	1.69	17.2
B35	-	-		-	-		-	-		-	-	
B37	-	-		1.41	5.54	25.5	5.90	19.9	29.6	4.72	17.0	26.0
B44	-	-		-	-		0.13	*23.3	*14.6	.47	3.17	14.8
B56	-	-		-	-		.45	3.02	14.9	.83	4.49	18.5
B32	-	-		*17.3	*14.4		1.40	5.37	26.1	1.32	4.62	28.6
B38	-	-		-	-		-	-		-	-	
B46	-	-		.22	1.19	18.5	1.07	4.22	25.4	.83	3.83	21.7
B47	* 7.0	* 5.9		1.48	6.04	24.5	1.98	7.92	25.0	1.86	5.94	31.3
B48	-	-		-	-		.13	*25.4	*13.6	.50	2.77	18.0
B49	-	-		* 5.5	* 4.5		.68	4.36	15.6	1.11	4.88	22.7
B42	-	-		-	-		* 8.9	* 6.8		.26	1.32	19.7
B43	-	-		-	-		.32	1.98	16.2	.42	2.77	15.2
B45	-	-		-	-		-	-		-	-	
B53	-	-		-	-		-	-		* 5.3	* 4.1	
B33	* 8.9	* 5.7		1.76	6.20	28.4	1.48	4.75	31.2	1.86	5.81	32.0
B41	*24.5	*20.1		4.17	16.24	25.7	4.47	16.24	27.5	5.50	16.63	33.1
B54	-	-		.33	1.58	20.9	.63	3.30	19.1	0.47	2.24	21.0
B55	.14	*25.1	*18.3	2.11	8.71	24.2	2.11	7.11	29.6	2.52	7.26	34.7
B57	-	-		*17.0	*10.6		.35	1.40	25.0	.31	1.04	29.8
B58	-	-		.40	2.38	16.8	.78	4.17	18.7	.61	3.30	18.5
B59	-	-		.37	1.85	20.0	*22.8	*20.7		1.11	3.80	29.2
B60	-	-		.40	1.58	25.3	*14.5	*13.8		.48	1.85	25.9
B61	-	-		.50	3.51	14.3	1.86	8.71	21.4	2.11	7.66	27.5
B50	-	-		.33	1.85	17.8	1.58	6.32	25.0	1.57	5.28	29.7

* Indicates the % binding of a1:10 dilution of antiserum or the estimation of the effect of dilution % from the % binding of a 1:10 dilution of antiserum; N.T., serum not tested.

Table 3.2 (contd.)

	Day 18			Day 21			Day 30		
	.02	.20	%	.02	.20	%	.02	.20	%
B30	.88	4.17	21.0	1.20	4.22	28.4	1.41	3.51	40.2
B39	1.41	4.58	30.8	1.76	4.62	38.1	1.78	3.95	44.9
B40	1.98	6.18	32.0	2.46	6.20	39.7	3.72	6.86	54.2
B34	.42	2.48	16.9	.32	1.58	20.3	0.20	⁎22.4	⁎ 22.8
B35	⁎ 6.0	⁎ 4.5		-	-		-	-	
B37	2.63	13.9	18.9	2.05	7.13	28.8	NT	NT	
B44	.44	2.64	16.7	.35	1.72	20.3	NT	NT	
B56	.58	3.63	16.0	.62	3.13	19.8	NT	NT	
B32	1.52	4.49	33.9	1.86	5.41	34.4	3.17	5.90	53.8
B38	⁎ 7.5	⁎ 4.9		⁎17.3	⁎10.9		⁎ 9.5	⁎ 4.1	
B46	.61	2.77	22.0	.59	2.38	24.8	.47	1.23	38.2
B47	2.35	5.94	39.6	2.96	7.66	38.6	3.63	6.60	55.0
B48	.44	2.11	20.9	.40	1.76	22.7	.36	⁎30.2	⁎27.5
B49	1.04	3.56	29.2	.95	3.63	26.2	1.11	3.17	35.0
B42	.22	⁎27.5	⁎17.7	.17	⁎20.4	⁎21.2	⁎28.9	⁎10.9	
B43	.42	2.11	19.9	.40	1.85	21.6	⁎ 7.3	⁎ 9.7	
B45	-	-		-	-		-	-	
B53	⁎24.9	⁎18.8		⁎28.3	⁎18.4		⁎20.4	⁎11.8	
B33	2.64	7.13	37.0	3.72	8.82	42.2	2.14	4.88	43.9
B41	6.24	16.24	38.4	6.24	15.71	39.7	7.96	14.12	56.4
B54	.42	1.41	29.8	.35	⁎27.5	⁎29.1	.12	⁎12.7	⁎32.8
B55	2.35	6.60	35.6	2.30	5.77	40.0	1.95	4.46	44.4
B57	.26	⁎23.6	⁎25.7	.22	⁎19.2	⁎27.9	.10	⁎11.7	⁎30.1
B58	.53	2.64	20.1	.48	2.14	22.4	.44	⁎28.7	⁎37.1
B59	1.00	3.43	29.2	.74	2.77	26.7	.44	1.45	36.3
B60	.36	1.15	31.3	.30	⁎26.1	⁎24.2	.22	⁎14.7	⁎37.8
B61	3.51	11.09	31.7	3.72	11.22	33.2	6.08	14.52	41.5
B50	1.95	5.41	36.0	1.86	5.28	35.2	1.70	3.70	45.9

Table 3.3: Secondary Response Data of Experiment 1

	Day 3			Day 6			Day 9		
	.02	.20	%	.02	.20	%	.02	.20	%
B30	44.2	44.2	100	525	603	87.1	351	496	70.8
B39	14.6	22.2	65.8	332	496	10.0	209	313	66.8
B40	115	155	74.2	836	1024	81.3	725	795	91.2
B34	* 7.1	* 5.3		11.1	25.8	42.6	9.34	24.8	37.7
B35	-	-		-	-		*11.8	* 6.8	
B37	27.9	28.9	96.5	107	105	100	97.9	102	95.9
B44	16.6	27.9	59.5	132	148	89.2	88.2	118	74.7
B56	31.7	31.3	100	81.4	100	81.4	62.5	61.8	100
B32	50.2	50.2	100	625	742	84.2	514	590	87.1
B38	-	-		* 11.0	* 9.3		*12.3	* 8.4	
B46	*12.2	*10.2		17.8	39.9	44.6	12.3	264	46.6
B47	105	126	83.3	677	709	95.5	468	632	74.1
B48	*21.9	*17.3		309	632	40.9	20.0	43.7	45.7
B49	2.95	6.90	50.0	223	351	63.5	125	209	54.8
B42	-.17	-		* 17.8	* 13.4		.78	*21.2	*27.4
B43	.50	2.24	223	59.0	118	50.0	47.9	97.8	49.0
B45	-	-		-	-		-	-	
B53	-	-		2.48	8.30	30.0	3.51	13.2	26.6
B33	D								
B41	222	223	100	1480	1550	95.5	9.34	872	100
B54	*16.4	*11.6		5.90	13.2	44.7	3.51	7.39	47.5
B55	69.3	76.0	91.2	490	557	88.0	332	372	89.2
B57	*23.7	*16.5		26.3	42.8	61.4	13.2	26.9	49.0
B58	8.92	12.3	72.5	111	159	69.8	62.5	118	59.2
B59	9.34	11.2	83.4	139	157	88.5	79.5	118	67.4
B60	.47	1.32	35.6	74.2	111	66.8	66.2	93.4	71.0
B61	55.7	81.4	58.4	191	235	81.2	145	191	75.9
B50	D								

* Indicates the % binding of a 1:10 dilution of antiserum or the estimation of the effect of dilution % from the % binding of a 1:10 dilution of antiserum; N.T., serum not tested; D indicates the death of a rabbit.

Table 3.3 (contd.)

	Day 15			Day 21			Day 30		
	.02	.20	%	.02	.20	%	.02	.20	%
B30	155	191	81.2	88.2	118	74.7	37.2	55.7	66.8
B39	934	148	63.1	55.0	69.3	79.4	23.5	34.7	67.7
B40	317	372	85.2	123	155	79.4	78.6	73.5	84.1
B34	3.89	13.9	28.0	2.79	6.86	40.7	1.70	3.56	47.8
B35	0.17	* 18.5	* 22.4	* 25.9	* 11.5		-	-	
B37	59.0	44.7	100	21.9	28.2	78.0	NT	NT	
B44	52.5	63.2	83.0	27.0	35.5	76.0	NT	NT	
B56	27.9	40.0	69.8	21.4	27.6	77.5	NT	NT	
B32	249	263	94.7	98.0	125	78.4	51.3	64.7	79.3
B38	* 19.6	* 15.0		* 13.9	* 8.4		-	-	
B46	11.1	23.5	47.2	4.00	8.32	48.0	1.86	3.70	50.2
B47	289	332	87.0	105	142	73.9	83.3	89.2	93.4
B48	13.2	27.9	47.3	8.33	19.9	41.9	5.38	12.3	43.7
B49	67.7	118	57.3	24.0	39.9	60.2	11.1	19.8	56.1
B42	1.11	2.90	38.3	.79	1.90	41.6	.42	* 22.9	* 47.5
B43	18.7	44.2	42.3	13.2	28.0	47.1	8.45	18.6	45.4
B45	-	-		-	-		-	-	
B53	2.35	7.00	33.6	1.58	5.0	31.6	.74	2.64	28.0
B33									
B41	525	557	84.3	263	209	100	129	126	100
B54	1.91	3.96	48.2	.79	2.38	33.2	.72	* 23.4	* 40.1
B55	222	235	94.5	85.20	112.00	76.7	52.50	62.40	84.1
B57	7.00	15.84	44.3	3.47	7.59	45.7	1.99	4.62	43.1
B58	27.9	55.0	50.7	13.2	24.0	55.0	7.96	15.2	52.4
B59	49.6	77.7	63.8	31.3	39.5	79.2	20.9	26.4	79.2
B60	15.7	29.6	53.0	9.78	14.5	67.4	3.55	5.90	60.2
B61	74.2	125	59.4	52.5	85.2	61.6	33.8	55.7	60.1
B50									

Experiment 2

The Effects of the Physical State of the Antigen and of *Corynebacterium parvum* on the Quantity and Quality of the Antibodies produced to Intraperitoneal Injection of Bovine Serum Albumin in Rabbits.

The objectives of the second experiment were primarily the same as for the first experiment; however, the experiment was modified in two ways. First the bovine albumin antigens were injected by the intraperitoneal route because it would be anticipated that the distribution of the antigen in the lymphoid tissue would be different than when the intravenous route was used. It is known, in many cases, that the intraperitoneal route is inferior to the intravenous route with respect to humoral antibody production to soluble protein antigens. Dresser (1962) used the intraperitoneal route to induce unresponsiveness in adult mice to BGG. The importance of the route of administration of antigen was shown by Battisto and Miller (1962) who demonstrated that adult guinea pigs could be more easily rendered unresponsive to BGG if the antigen was injected into the mesenteric vein. The second modification was designed to test the temporal relationship between the administration of *C. parvum* and the injection of the bovine antigens. It was decided to use only CBA and ACBA as antigen and to inject *C. parvum* either 6 days prior to or simultaneously with the respective bovine antigens. The design of the experiment is shown in Table 3.4.

Table 3.4

Design of Experiment 2

#	Sex	Wt.(Kg)	Pretreatment Day - 6	Primary Day 0	Secondary Day 30
B6	F	3.0	15 mg *C. parvum* I.V.	50 mg ACBA I.P.	10 mg ACBA I.P.
B7	M	2.7			
B0	F	3.0	None	50 mg ACBA I.P. 15 mg *C. parvum* I.V.	10 mg ACBA I.P.
B1	M	2.6			
B8	M	2.9	15 mg *C. parvum* I.V.	50 mg CBA I.P.	10 mg CBA I.P.
B9	F	2.5			
B2	F	2.5	None	50 mg CBA I.P. 15 mg *C. parvum* I.V.	10 mg CBA I.P.
B3	M	2.7			
B11	M	3.0			
B12	M	3.1			
B13	F	2.6			

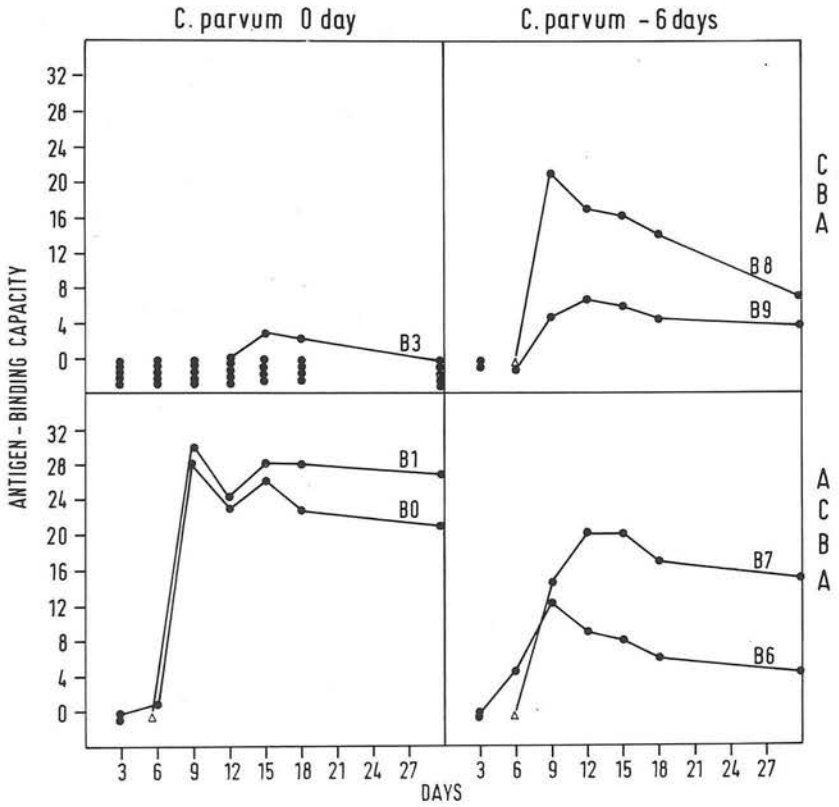
Primary response

Three month old New Zealand White rabbits of both sexes were used. Four parallel groups were run, two groups of rabbits injected with 15 mg of a heat-killed suspension of C. parvum 6 days prior to the intraperitoneal injection of either 50 mg CBA or 50 mg ACBA. The two remaining groups were injected simultaneously with 15 mg of C. parvum into the marginal ear vein and either 50 mg of CBA or 50 mg of ACBA intraperitoneally. The results of the primary responses are shown in Figure 3.5, which reflects the levels of anti-BSA antibodies produced, and in Figure 3.6 which shows the increase in the relative binding affinity of the antibodies. More detailed information is given in Table 3.5.

Antigen-binding capacity: The results expressed in Figure 3.5 show that rabbits, either given C. parvum on day -6 and CBA or ACBA on day 0 or C. parvum and ACBA on day 0, elicited a strong primary antibody response as compared to rabbits given C. parvum and CBA on day 0.

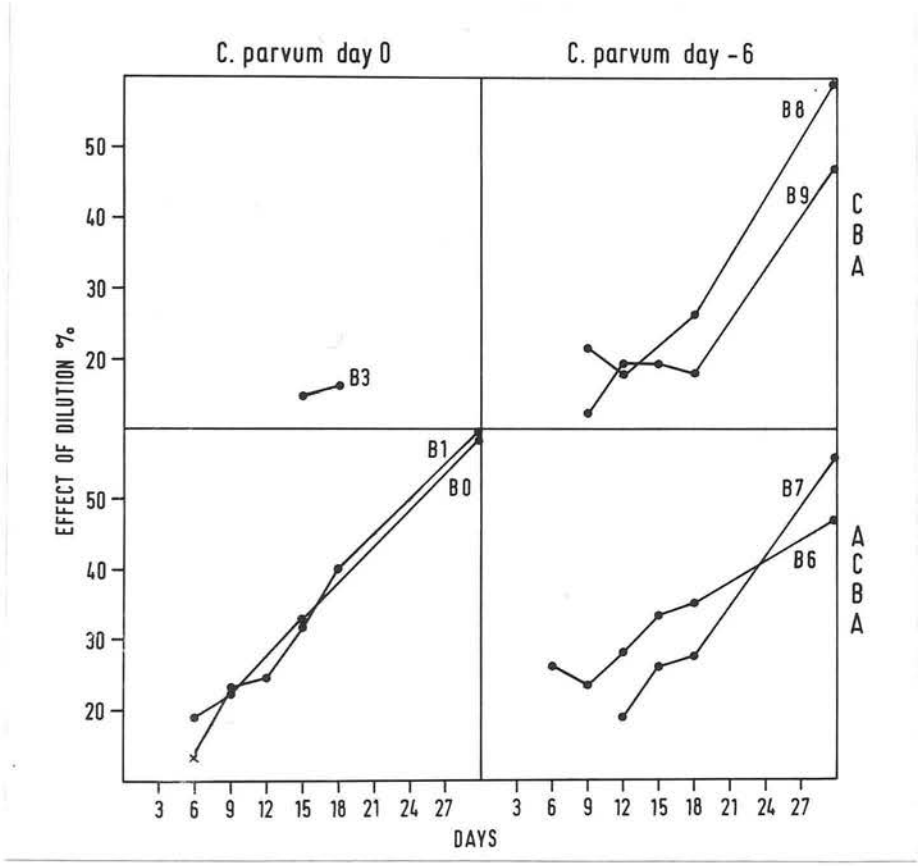
Effect of dilution: The results shown in Figure 3.6 indicate that in all of the groups, with the exception of the rabbits receiving C. parvum and CBA on day 0, there is a constant, rapid increase in the effect of dilution percentage values reflecting accelerated evolution of the relative binding affinity of the antibodies produced. On days 15 and 18 when ABCs could be determined for rabbit B 3, the effect of dilution percentage of the antisera from this rabbit were lower than those in rabbits from the other three groups.

Figure 3.5



Primary response: Antigen-binding capacity (Reciprocal ABC-33 end point x 0.20 ug N BSA x 2 x 0.33) of Corynebacterium parvum (day 0) and C. parvum (-6 days) treated groups given intraperitoneal injections of centrifuged bovine albumin (CBA) or alum-precipitated centrifuged bovine albumin (ACBA) on day 0. Δ, insufficient antibody to give a positive ABC at a 1:10 dilution of antiserum.

Figure 3.6



Primary response: Effect of dilution % ((ABC-33, 0.02 ug N BSA/ABC-33, 0.20 ug N BSA) x 100) of Corynebacterium parvum (day 0) and C. parvum (-6 days) treated groups given intraperitoneal injections of CBA or ACBA on day 0. X indicates the estimated effect of dilution % from the % binding of a 1:10 dilution of antiserum.

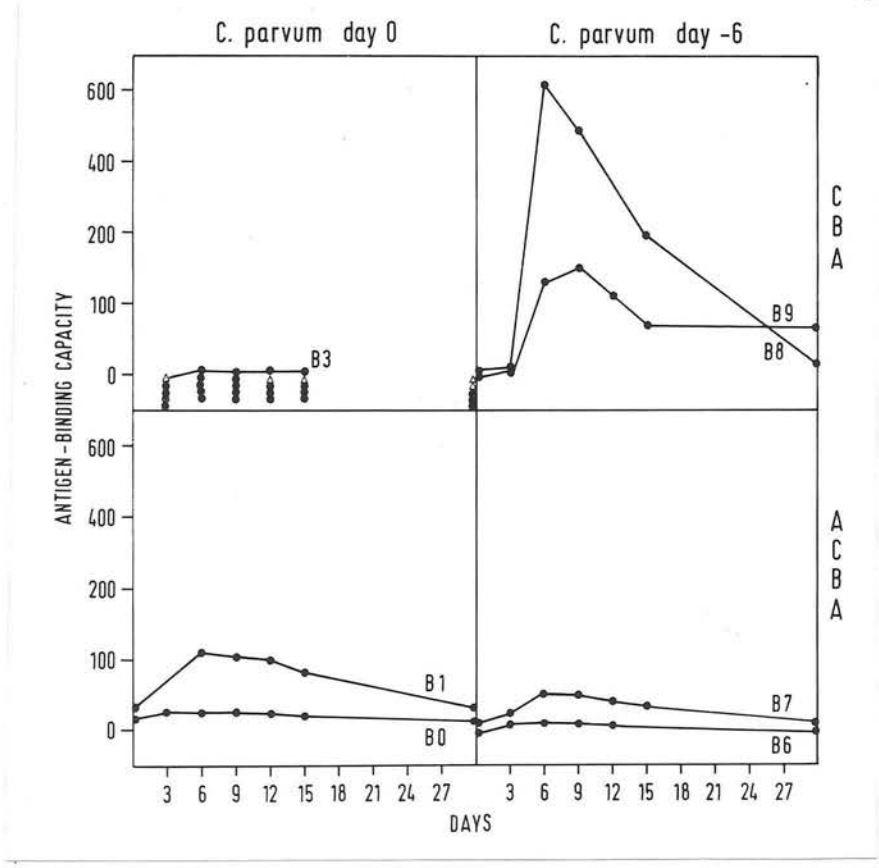
Secondary response

On day 30 of the primary response, all of the rabbits were given intraperitoneal injections of 10 mg of the bovine albumin antigens in the same physical form as they received for the primary stimulus. The decreased amount of antigen, compared with that given for the primary stimulus, was used in the hope of providing a more sensitive measure of the degree of primary stimulation. None of the rabbits were injected with C. parvum before the secondary response. The results of the secondary responses are shown in Figure 3.7 and Figure 3.8 which indicate the magnitude of the ABC values and effect of dilution percentage values respectively. More detailed information is given in Table 3.6.

Antigen-binding capacity: The results of the ABC values shown in Figure 3.7 were not as had been anticipated. The groups injected with ACBA did not elicit the expected high anamnestic response, whereas both rabbits which received C. parvum on day -6 and CBA on day 0 of the primary response, gave a strong secondary response when challenged with CBA. The remaining group which received C. parvum and CBA on day 0 was hyporesponsive or unresponsive after challenging with CBA on day 30.

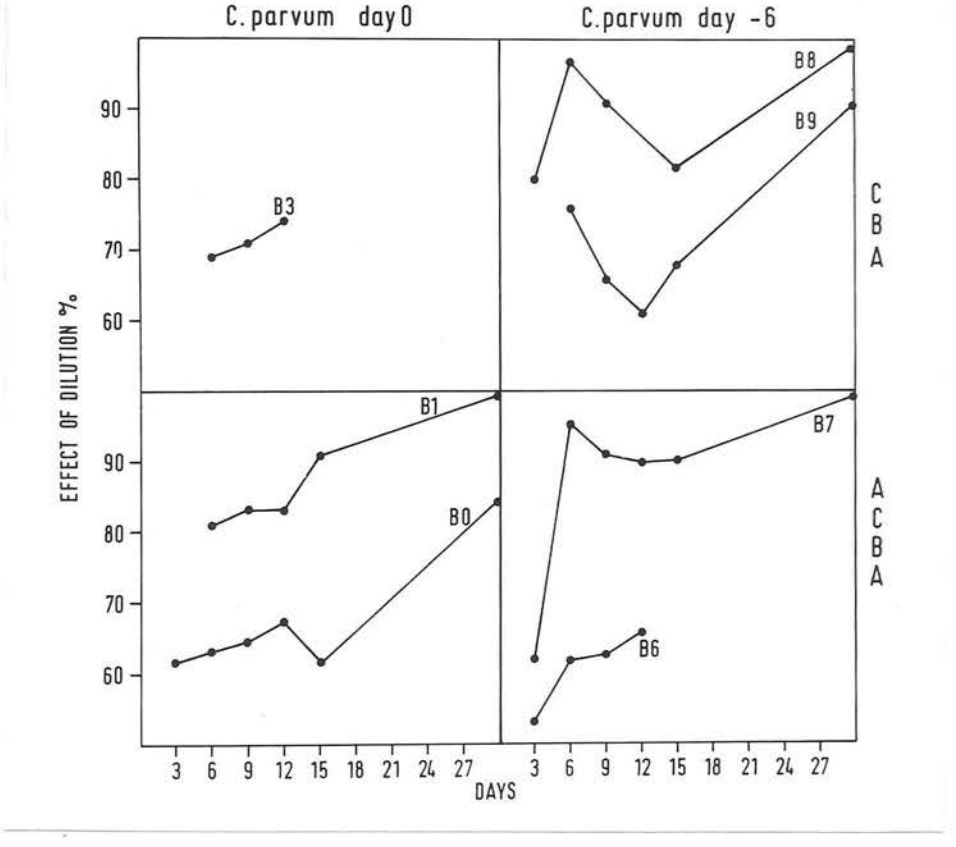
Effect of dilution: The results of the effect of dilution percentage are shown in Figure 3.8. In all of the groups there was in general an immediate, rapid increase in the effect of dilution percentage values. Little comment can be made in comparing the groups as the number of rabbits responding in

Figure 3.7



Secondary response: Antigen-binding capacity (Reciprocal ABC-33 end point x 0.20 ug N BSA x 2 x 0.33) of Corynebacterium parvum (day 0) and C. parvum (-6 days) treated groups given secondary intraperitoneal injections of CBA or ACBA on day 30 of the primary response (i.e., day 0 of the secondary response). Δ,insufficient antibody to give a positive ABC at a 1:10 dilution.

Figure 3.8



Secondary response: Effect of dilution % ((ABC-33, 0.02 ug N BSA/ABC-33, 0.20 ug N BSA) x 100) of Corynebacterium parvum (day 0) and C. parvum (-6 days) treated groups given secondary intraperitoneal injections of CBA or ACBA on day 30 of the primary response (i.e., day 0 of the secondary response).

each group is small.

Conclusion

The intravenous injection of C. parvum simultaneously with an intraperitoneal injection of CBA does not produce the hyperreactive state as does C. parvum when injected 6 days prior to the intraperitoneal injection of CBA. Whereas particulate ACBA has been shown to be more effective than CBA in inducing a primary antibody response, the reverse was true for secondary stimulation by the intraperitoneal route.

Table 3.5: Primary Response Data of Experiment 2

	Day 6			Day 9			Day 12		
	.02	.20	%	.02	.20	%	.02	.20	%
B 6	1.17	4.36	26.8	2.96	12.6	23.5	2.61	9.37	27.9
B 7	*11.0	* 8.7		NT	15.8		3.72	19.9	18.7
B 0	.18	.94	19.1	6.27	27.6	22.6	NT	23.4	
B 1	*23.6	*17.8		7.01	30.2	23.2	6.03	24.6	24.5
B 8	*16.4	*18.4		4.58	21.4	21.4	2.96	17.0	17.4
B 9	-	-		.55	4.58	12.0	1.27	6.60	19.2
B 2	-	-		-	-		-	-	
B 3	-	-		-	-		-	-	
B11	-	-		-	-		-	-	
B12	-	-		-	-		-	-	
B13	-	-		-	-		-	-	

* Indicates the % binding of a 1:10 dilution of antiserum or the estimation of the effect of dilution % from the % binding of a 1:10 dilution of antiserum; NT, serum not tested.

Table 3.5 (contd.)

	Day 15			Day 18			Day 30		
	.02	.20	%	.02	.20	%	.02	.20	%
B 6	2.79	8.32	33.5	2.19	6.24	35.1	1.95	4.17	46.8
B 7	5.13	19.3	25.7	4.79	17.4	27.5	8.45	15.2	55.7
B 0	8.71	26.3	33.1	NT	22.9		12.6	21.5	58.6
B 1	8.92	27.9	32.0	11.2	28.3	39.8	16.2	26.9	60.3
B 8	NT	16.4		3.72	14.0	26.6	5.25	7.59	69.2
B 9	1.12	5.89	19.0	.74	4.36	16.7	1.86	3.95	47.0
B 2	-	-		-	-		-	-	
B 3	.42	2.90	14.4	.37	2.35	15.7	-	-	
B11	-	-		-	-		-	-	
B12	-	-		-	-		-	-	
B13	-	-		-	-		-	-	

Table 3.6: Secondary Response Data of Experiment 2

	Day 3			Day 6			Day 9		
	.02	.20	%	.02	.20	%	.02	.20	%
B 6	2.22	4.17	53.2	3.13	5.02	61.8	3.30	5.28	62.5
B 7	13.3	21.4	62.1	47.9	50.2	95.4	44.7	49.1	91.0
B 0	15.5	25.2	61.5	15.9	25.2	63.1	16.5	25.7	64.2
B 1	NT	NT		89.2	110	81.1	81.2	105	83.0
B 8	7.77	9.77	79.5	625	646	96.7	447	490	91.2
B 9	-	-		132	174	75.9	99.0	151	65.6
B 2	-	-		-	-		-	-	
B 3	.44	*21.0	*51.0	2.05	2.96	69.0	2.35	3.30	71.2
B11	-	-		-	-		-	-	
B12	-	-		-	-		-	-	
B13	-	-		-	-		-	-	

* Indicates the % binding of a 1:10 dilution of antiserum or the estimation of the effect of dilution % from the % binding of a 1:10 dilution of antiserum; NT, serum not tested.

Table 3.6 (contd.)

	Day 12			Day 15			Day 30		
	.02	.20	%	.02	.20	%	.02	.20	%
B 6	3.30	5.02	65.7	NT	NT		-	-	
B 7	37.2	41.8	88.9	32.1	35.5	90.4	3.46	3.43	100
B 0	15.9	23.5	67.5	13.5	21.9	61.6	11.8	14.1	83.7
B 1	82.5	99.0	83.3	72.5	99.6	91.1	33.1	33.3	100
B 8	NT	NT		118	197	82.2	46.8	63.2	63.2
B 9	67.7	111	61.0	46.9	69.3	67.7	15.5	17.6	74.0
B 2	.13	*13.5	*24.8	.26	*25.4	*33.1	.20	* 9.6	*55.0
B 3	2.34	3.17	73.8	1.86	2.44	76.2	1.04	*25.0	*83.1
B11	-	-		-	-		-	-	
B12	-	-		-	-		-	-	
B13	-	-		-	-		-	-	

Experiment 3

The Effect of *Corynebacterium parvum* on the Induction of Immunological Unresponsiveness to Bovine Serum Albumin in Adult Rabbits by Intravenous Injection of Small Amounts of Centrifuged Bovine Albumin.

The first two experiments demonstrated that the injection of CBA into normal adult rabbits, rendered these animals hyporesponsive to CBA even after further challenge. Although a number of normal rabbits treated with CBA did not produce any detectable anti-BSA antibody, it was decided to design an experiment which would specifically demonstrate whether these rabbits were rendered unresponsive to bovine albumin or whether they were simply not immunized. Therefore, rabbits were given intravenous injections of CBA for the primary stimulus and challenged 30 days later with intravenous injections of ACBA. ACBA is a powerful antigen and, in every case, has elicited a vigorous primary response in adult normal rabbits; thus this procedure would provide means to test for the unresponsive state. The adjuvant effect of *C. parvum* was also tested by either injecting the organism 6 days prior to or simultaneously with CBA. The design of the experiment is shown in Table 3.7.

Primary response

Three to four month old New Zealand White rabbits of both sexes were used. Three parallel groups were run. The first

Table 3.7

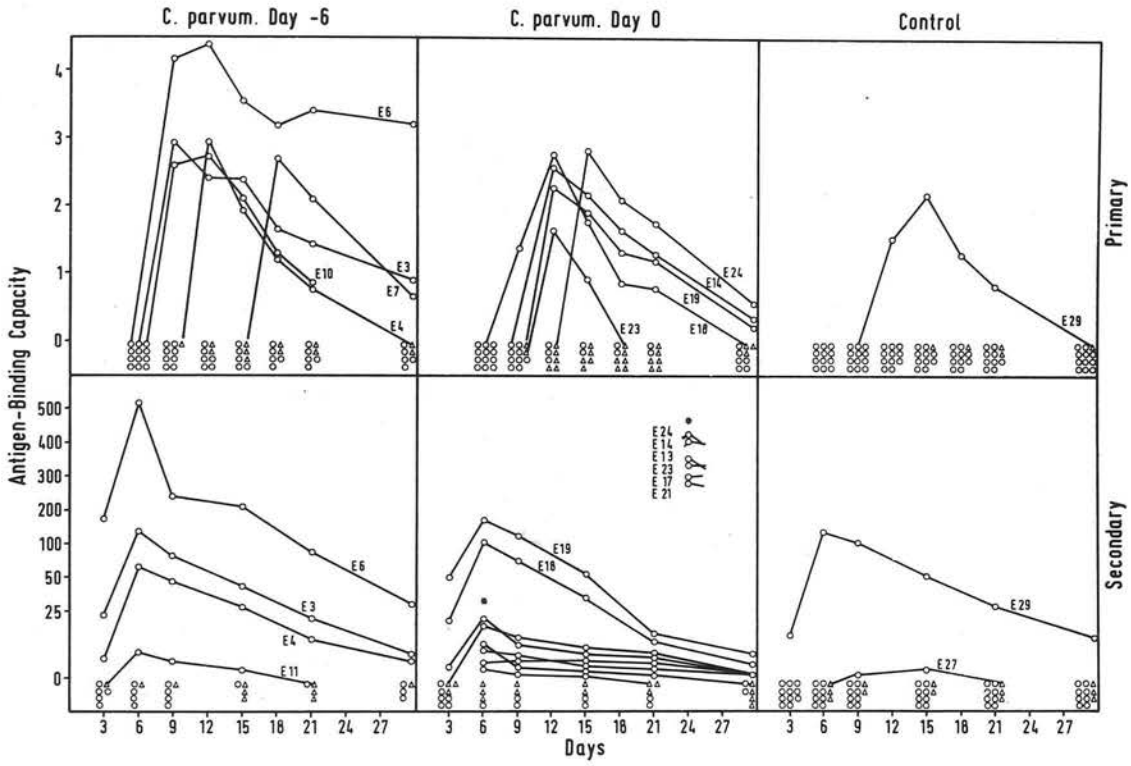
Design of Experiment 3

#	Sex	Wt.(kg)	Pretreatment Day - 6	Primary Day 0	Secondary Day 30
E1	M	3.5	15 mg *C. parvum* I.V.	10 mg CBA I.V.	25 mg ACBA I.V.
E2	M	3.3			
E3	M	3.0			
E4	M	2.9			
E5	M	2.8			
E6	M	2.5			
E7	F	3.5			
E8	F	3.3			
E9	F	3.2			
E10	F	3.0			
E11	F	2.9			
E12	F	2.4			
E13	M	3.5	None	10 mg CBA I.V. & 15 mg *C. parvum* I.V.	25 mg ACBA I.V.
E14	M	3.2			
E15	M	3.0			
E16	M	2.8			
E17	M	2.7			
E18	M	2.5			
E19	M	1.9			
E20	F	3.4			
E21	F	3.3			
E22	F	3.2			
E23	F	2.8			
E24	F	2.5			
E25	M	3.3	None	10 mg CBA I.V.	25 mg ACBA I.V.
E26	M	3.0			
E27	M	2.9			
E28	M	2.8			
E29	M	2.6			
E30	M	2.5			
E31	F	3.4			
E32	F	3.2			
E33	F	3.0			
E34	F	2.9			
E35	F	2.8			
E36	F	2.6			

group was injected intravenously with 15 mg of a heat-killed suspension of C. parvum 6 days prior to the intravenous injection of 10 mg CBA on day 0. The second group was injected in the left marginal ear vein with 10 mg(1.66 mg N) CBA followed 15 minutes later by an injection of 15 mg C. parvum into the right marginal ear vein. The third group received intravenous injections of 10 mg CBA on day 0. The results of the primary response are shown in Figure 3.9 which reflects the levels of anti-BSA antibodies produced. The results of the effect of dilution percentage and more detailed information are given in Table 3.8.

Antigen-binding capacity: The results expressed in Figure 3.9 show that the rabbits injected with 10 mg CBA on day 0 in general did not produce anti-BSA antibodies. E 29 was the only rabbit out of twelve to produce enough anti-BSA antibodies to give positive ABC values; one or two other rabbits in this group appeared to produce antibodies but the amounts were negligible. The remaining nine rabbits did not produce detectable anti-BSA antibody. The rabbits which received C. parvum 6 days prior to CBA responded to a greater degree than the previous group. Five out of twelve rabbits produced ample antibody levels to give positive ABC values and two more rabbits produced antibody just below the minimum amount required to give a positive ABC. Five rabbits in this group did not produce detectable antibody. The third group, which received C. parvum and CBA on day 0, was similar to the group just described. Five out of twelve rabbits produced

Figure 3.9



Primary response: Antigen-binding capacity (Reciprocal ABC-33 end point x 0.20 ug N BSA x 2 x 0.33) of Corynebacterium parvum (-6 days), C. parvum (day 0) treated and control groups given intravenous injections of centrifuged bovine albumin (CBA) on day 0. Δ, insufficient antibody to give a positive ABC at a 1:10 dilution of antiserum.

enough antibody to give positive ABC values. Five rabbits produced anti-BSA antibody below the amount needed to give a positive ABC; two rabbits did not produce detectable antibody.

Effect of dilution: Little comment can be made as only one rabbit in the CBA control group produced enough antibody to enable the estimation of the effect of dilution percentage as seen in Table 3.8. The effect of dilution percentage values in the other two groups were similar, indicating a rapid increase in the relative binding affinity of the antibodies produced.

Secondary response

On day 30 of the primary response, all of the surviving rabbits were given intravenous injections of 25 mg ACBA. None of the rabbits were injected with C. parvum before the secondary stimulus. The results of the secondary responses are shown in Figure 3.10 which reflects the levels of anti-BSA antibodies produced. The results of the effect of dilution percentage and more detailed information are given in Table 3.9.

Antigen-binding capacity: The results of the secondary responses shown in Figure 3.10 demonstrate that eight out of twelve rabbits, which had received 10 mg CBA for the primary stimulus, were rendered unresponsive to BSA. Two rabbits produced sufficient antibody to give positive ABC values. The two remaining rabbits in this group produced only small amounts of antibody and ABC values could not be determined. The results of the other two groups differ significantly from the

Figure 3.10

See Figure 3.9

Secondary response: Antigen-binding capacity (Reciprocal ABC-33 end point x 0.20 ug N BSA x 2 x 0.33) of Corynebacterium parvum (-6 days), C. parvum (day 0) treated and control groups given secondary intravenous injections of alum-precipitated centrifuged bovine albumin (ACBA) on day 30 of the primary response (i.e., day 0 of the secondary response). Δ, insufficient antibody to give a positive ABC at a 1:10 dilution.

group just described. Eight out of twelve rabbits, which had received 15 mg C. parvum and 10 mg CBA on day 0, gave positive ABC values after secondary challenge with ACBA. Two rabbits produced low levels of anti-BSA antibodies and two were unresponsive. The results of the remaining group were more difficult to interpret due to deaths which occurred towards the end of the primary response and the early secondary response. Sera from four of the surviving rabbits had positive ABC values and it could be postulated that rabbits E 7 and E 10 would also have produced high levels of antibody in the secondary response. Four rabbits were unresponsive after secondary challenge with ACBA and two rabbits produced low levels of anti-BSA antibodies. It would therefore appear that both groups of rabbits which received C. parvum before or together with CBA, were similar with respect to number of rabbits responding and the magnitude of the ABC values.

Effect of dilution: As in the primary response little can be said about the effect of dilution percentage in the secondary response. The relative binding affinity of the antibodies in all of the groups generally rose to levels above that attained in the primary response (see Table 3.9).

Conclusion

The intravenous injection of 10 mg CBA into normal adult rabbits induced an unresponsive state in two-thirds of the rabbits; one quarter of the rabbits became partially tolerant and one twelfth of the rabbits responded to CBA. In contrast

only one sixth of the rabbits which were injected with C. parvum either 6 days prior to or simultaneously with 10 mg of CBA were rendered unresponsive to BSA; one-half of the rabbits responded to CBA and one-third of the rabbits became partially tolerant to BSA.

Table 3.8: Primary Response Data of Experiment 3

	Day 6			Day 9			Day 12			Day 15		
	.02	.20	%	.02	.20	%	.02	.20	%	.02	.20	%
E 1	-	-		-	-		⁎11.4	⁎ 5.1		.30	⁎24.8	⁎30.2
E 2	-	-		-	-		-	-		-	-	
E 3	-	-		.76	2.96	25.7	.66	2.38	27.7	.60	2.35	25.5
E 4	-	-		⁎8.1	⁎6.3		.43	2.96	14.5	.37	1.99	18.6
E 5	-	-		-	-		-	-	-	-	-	
E 6	-	-		.91	4.17	21.8	1.04	4.36	23.9	.96	3.56	27.0
E 7	-	-		-	-		-	-		.20	⁎25.1	⁎24.7
E 8	-	-		-	-		-	-		-	-	-
E 9	-	-		-	-		-	-		-	-	-
E10	-	-		.50	2.69	18.6	.59	2.76	21.4	.43	2.10	20.5
E11	-	-		-	-		.18	⁎20.4	⁎21.2	.18	⁎21.4	⁎20.1
E12	-	-		-	-		-	-	-	-	-	
E13	-	-		-	-		⁎24.7	⁎ 9.3		.25	⁎19.1	⁎25.7
E14	-	-		-	-		.55	2.67	20.8	.53	2.22	23.9
E15	-	-		-	-		-	-		-	-	
E16	-	-		-	-		-	-		⁎14.7	⁎ 7.3	
E17	-	-		-	-		⁎13.2	⁎ 8.1		.26	⁎26.0	⁎24.6
E18	-	-		.31	1.41	22.0	.73	2.77	26.4	.43	1.82	23.6
E19	-	-		⁎4.7	⁎3.8		.40	2.35	17.0	.38	1.93	19.7
E20	-	-		-	-		-	-	-	-	-	
E21	-	-		-	-		⁎ 8.1	⁎ 6.5		.21	⁎25.1	⁎18.0
E22	-	-		-	-		⁎ 6.5	⁎ 5.0		⁎28.9	⁎17.0	⁎17.6
E23	-	-		⁎4.8	⁎4.0		.33	1.66	19.9	.28	⁎29.1	⁎22.3
E24	-	-		-	-		⁎16.0	⁎11.4		.50	2.82	17.7
E25	-	-		-	-		-	-		-	-	
E26	-	-		-	-		-	-		-	-	
E27	-	-		-	-		-	-		⁎14.2	⁎ 7.4	
E28	-	-		-	-		-	-		-	-	
E29	-	-		-	-		.31	1.56	19.9	.47	2.19	21.5
E30	-	-		-	-		-	-		⁎ 6.1	⁎ 3.8	
E31	-	-		-	-		-	-		-	-	
E32	-	-		-	-		-	-		-	-	
E33	-	-		-	-		-	-		-	-	
E34	-	-		-	-		-	-		-	-	
E35	-	-		-	-		-	-		-	-	
E36	-	-		-	-		-	-		-	-	

⁎ Indicates the % binding of a 1:10 dilution of antiserum or the estimation of the effect of dilution % from the % binding of a 1:10 dilution of antiserum; D indicates the death of a rabbit.

Table 3.8 (contd.)

	Day 18			Day 21			Day 30		
	.02	.20	%	.02	.20	%	.02	.20	%
E 1	.32	⁎19.8	x36.5	.21	⁎15.8	⁎32.3	⁎18.5	⁎ 4.5	
E 2	D	-		-	-			D	
E 3	.55	1.66	33.1	.50	1.45	34.5	.46	⁎29.3	⁎44.8
E 4	.36	1.32	27.3	.28	⁎29.0	⁎25.7	.20	⁎16.2	⁎28.2
E 5	-	-		-	-		-	-	
E 6	1.07	3.17	33.8	1.11	3.39	32.7	1.39	3.17	43.8
E 7	.76	2.69	28.3	.66	2.10	31.4	.36	⁎24.8	⁎41.4
E 8	-	-		-	-		-	-	
E 9	-	-		-	-		-	-	
E10	.40	1.32	30.2	.30	⁎26.4	⁎27.2	D		
E11	.13	⁎13.2	⁎29.5	⁎19.6	⁎ 9.1		⁎10.4	-	
E12	-	-		-	-		-	-	
E13	.24	⁎15.9	⁎34.2	.15	⁎12.1	⁎29.0	⁎18.4	-	
E14	.47	1.66	28.3	.40	1.32	30.3	.26	⁎19.4	⁎34.1
E15	⁎14.7	⁎7.4		⁎10.4	⁎ 6.8		⁎ 6.3	-	
E16	.13	⁎9.2	⁎31.2	⁎30.6	⁎ 9.1	⁎33.0	⁎12.4	-	
E17	.44	⁎30.9	⁎34.8	.33	⁎25.4	⁎32.7	.18	⁎11.9	⁎40.2
E18	.13	⁎27.5	⁎35.7	.32	⁎24.1	⁎31.4	.44	⁎18.6	⁎55.7
E19	.40	1.32	30.0	.43	1.32	32.6	-.43	⁎23.0	⁎51.5
E20	-	-		-	-		-	-	
E21	.20	⁎17.1	⁎26.5	.13	⁎14.6	⁎22.7	⁎20.0	⁎ 6.7	
E22	⁎31.7	⁎10.6	⁎33.4	⁎21.4	⁎ 8.7		⁎ 6.6	-	
E23	.21	⁎18.5	⁎27.4	.13	⁎15.2	⁎21.2	⁎10.1	-	
E24	.48	2.11	22.7	.43	1.76	24.4	.29	⁎20.6	⁎34.6
E25	-	-		-	-		-	-	
E26	-	-		-	-		⁎10.8	-	
E27	⁎30.2	⁎ 8.2		⁎25.0	⁎ 8.1		⁎13. 2	-	
E28	-	-		-	-		-	-	
E29	.35	1.32	26.5	.31	⁎26.9	⁎26.7	.18	⁎14.2	⁎32.2
E30	⁎14. 1	⁎ 3.1		⁎15.3	⁎ 4.5		⁎ 6.5	-	
E31	⁎11.1	⁎ 4.6		⁎13.3	⁎ 7.9		⁎ 5.3	-	
E32	-	-	-	-	-		-	-	
E33	-	-		-	-		-	-	
E34	-	-		-	-		-	-	
E35	-	-		-	-		-	-	
E36	-	-		-	-		-	-	

Table 3.9: Secondary Response Data of Experiment 3

	Day 3			Day 6			Day 9		
	.02	.20	%	.02	.20	%	.02	.20	%
E 1	-	-		.24	*21.4	*20.1	.37	*23.0	*34.2
E 2									
E 3	13.2	21.4	61.7	99.0	140	70.7	66.1	81.4	81.4
E 4	4.28	7.59	56.4	40.8	66.1	61.7	25.7	46.9	57.8
E 5	-	-		-	-		-	-	
E 6	132	174	75.8	372	513	72.5	209	240	87.0
E 7	D								
E 8	-	-		-	-		-	-	
E 9	-	-		-	-		-	-	
E10									
E11	*11.5	* 6.3		4.47	9.90	45.2	3.39	6.73	50.4
E12	-	-		-	-		-	-	
E13	.44	*22.0	* 50.0	6.61	13.2	50.1	2.96	4.88	60.6
E14	*24.5	*13.5		11.8	21.9	53.9	9.34	16.2	57.7
E15	-	-		*25.3	*18.3		.24	*27.7	*32.7
E16	-	-		.20	*16.2	*38.3	.46	*28.4	*41.9
E17	*14.9	* 8.6		2.22	6.03	36.8	3.30	7.09	46.5
E18	13.2	21.0	62.9	76.0	107	71.0	55.7	74.2	75.0
E19	37.2	49.6	75.0	125	170	73.5	77.7	118	65.8
E20	-	-		-	-		-	-	
E21	-	-		1.86	3.70	50.3	1.13	3.20	35.3
E22	-	-		*13.1	* 5.9		*16.7	-	
E23	* -	-		4.26	10.0	42.6	3.30	8.14	40.5
E24	1.58	3.96	40.0	14.2	23.5	60.4	9.37	12.5	74.7
E25	-	-		-	-		-	-	
E26	-	-		-	-		-	-	
E27	-	-		.32	*25.9	*30.7	.88	1.51	58.1
E28	-	-		-	-		-	-	
E29	6.91	15.7	44.0	93.5	142	65.8	72.5	105	69.0
E30	-	-		*28.4	x 19.0		.33	*18.7	*57.2
E31	-	-		*17.4	* 8.9		.16	*18.1	*37.5
E32	-	-		-	-		-	-	
E33	-	-		-	-		-	-	
E34	-	-		-	-		-	-	
E35	-	-		-	-		-	-	
E36	-	-		-	-		-	-	

* Indicates the % binding of a 1:10 dilution of antiserum or the estimation of the effect of dilution % from the % binding of a 1:10 dilution of antiserum; D indicates the death of a rabbit.

Table 3.9 (contd.)

	Day 15			Day 21			Day 30		
	.02	.20	%	.02	.20	%	.02	.20	%
E 1	.42	*23.4	* 42.9	.14	* 9.8	*40.8	*20.1	-	
E 2									
E 3	40.8	42.8	95.3	20.5	23.0	89.1	7.96	9.50	83.8
E 4	14.2	27.9	50.9	7.96	15.2	52.4	3.55	7.79	45.6
E 5	-	-		-	-		-	-	
E 6	209	209	100	69.7	88.2	79.0	35.5	35.1	100
E 7									
E 8	* 7.0	* 6.5		D					
E 9	* 9.2	* 7.1		.14	*10.4	*41.2	-	-	
E10									
E11	1.74	3.80	45.8	.59	*27.8	*43.2	.26	*12.8	*43.7
E12	D								
E13	3.30	5.25	62.9	1.56	2.38	65.5	.62	1.45	42.8
E14	6.18	9.56	64.7	2.82	4.58	61.6	2.14	3.63	59.0
E15	.66	1.40	47.0	.50	*20.8	*52.7	.33	*13.6	*55.1
E16	.62	1.32	47.0	.32	*12.6	*54.2	.31	*12.3	*42.5
E17	5.92	7.77	76.2	3.39	4.47	75.8	2.30	3.38	68.0
E18	30.1	35.5	84.5	12.6	16.6	75.9	4.27	5.25	81.3
E19	52.5	52.5	100	18.7	18.6	100	10.7	10.7	100
E20	*14.9	* 5.0		*17.2	* 4.0		* 6.1	-	
E21	.72	2.08	34.6	1.10	*25.4	*47.3	.50	*20.9	*52.3
E22	*15.1	* 7.1		-	-		-	-	
E23	2.76	5.28	46.6	.85	1.98	42.9	.30	*17.9	*39.5
E24	6.23	8.71	71.3	4.15	5.94	70.0	2.19	3.43	64.0
E25	-	-		-	-		-	-	
E26	-	-		-	-		-	-	
E27	1.32	1.85	71.3	.66	*28.0	*67.5	.35	*18.8	*56.2
E28	-	-		-	-		-	-	
E29	39.0	52.5	74.0	22.4	29.5	76.3	10.5	16.6	63.3
E30	.59	*30.0	*50.0	.20	*10.1	*42.4	.21	*10.1	*49.3
E31	.32	*23.7	*35.0	.31	*14.5	*28.6	*23.8	* 7.9	*33.1
E32	-	-		-	-		-	-	
E33	-	-		-	-		-	-	
E34	-	-		-	-		-	-	
E35	-	-		-	-		-	-	
E36	-	-		-	-		-	-	

Experiment 4

The Effect of Corynebacterium parvum on the Induction of Immunological Unresponsiveness to Bovine Serum Albumin in Neonatal Rabbits.

The third experiment demonstrated that C. parvum interferes with the induction of immunological unresponsiveness to BSA in adult rabbits. This experiment was designed to determine whether C. parvum would inhibit the induction of immunological unresponsiveness in the neonatal rabbit by injecting a heat-killed suspension of C. parvum 6 days prior to the tolerance inducing injection of BSA.

Induction of unresponsiveness

Two groups of neonatal rabbits were employed. Eleven newborn New Zealand White rabbits were injected intraperitoneally with 2 mg of a heat-killed suspension of C. parvum; the C. parvum was injected less than 12 hours after birth. When the neonatal rabbits were 6 days old, they received intraperitoneal injections of 100 mg BSA. Ten other newborn rabbits were injected intraperitoneally with 100 mg BSA 6 days after birth. The rabbits were allowed to mature without further treatment until they were three months of age.

Testing the unresponsive state

All twenty-one rabbits were given intravenous injections of 50 mg NBA when they were three months old. They were bled

every third day, commencing nine days after the primary injection of NBA, until day 30 when a secondary injection of 50 mg NBA was given intravenously. The rabbits were bled every third day for another thirty days. The sera from the primary and secondary responses were tested for anti-BSA antibodies by the Farr technique, using 0.20 ug N BSA* and 0.020 ug N BSA*.

Primary and secondary responses

The ten control rabbits, which received 100 mg BSA 6 days after birth, did not produce detectable anti-BSA antibodies after the primary injection of NBA; the nine surviving rabbits from this group did not produce detectable anti-BSA antibodies after secondary stimulation with NBA. Of the eleven rabbits, which received 2 mg C. parvum twelve hours after birth and 100 mg BSA 6 days later, only one rabbit produced anti-BSA antibodies after the primary stimulation with NBA; however, the amount of antibody produced was not of sufficient quantity to determine ABC values. All but one of the surviving ten rabbits did not produce anti-BSA antibodies after secondary stimulation with NBA. The rabbit that responded was the same rabbit that responded to the primary stimulation with NBA and again the level of anti-BSA antibody was too low to determine ABC values.

Conclusion

C. parvum injected 6 days prior to BSA does not interfere with the induction of unresponsiveness in the neonatal rabbit.

Experiment 5

The Effect of *Corynebacterium parvum* on the Termination of Neonatally Induced Unresponsiveness to Bovine Serum Albumin in Three Month Old Adult Rabbits.

The fifth experiment was designed to test whether *C. parvum* would abrogate a pre-existing unresponsive state to BSA. Rabbits were rendered unresponsive to BSA by the injection of BSA during the neonatal period. At three months of age these rabbits received intravenous injections of *C. parvum* followed 6 days later by intravenous injections of NBA. The rabbits were bled every third day after the injection of NBA until day 30 when a second injection of NBA was given; the rabbits were then bled every third day for a further 30 days. The sera from the control and experimental rabbits were checked for anti-BSA antibodies by the Farr technique, using 0.20 ug N BSA* and 0.020 ug N BSA*.

Induction of unresponsiveness

Six neonatal New Zealand White rabbits, less than twelve hours old, were injected intraperitoneally with 100 mg BSA. The rabbits were allowed to mature without further treatment until they were three months of age.

Termination of unresponsiveness

When the rabbits were three months old, three were injected intravenously with 10 mg of a heat-killed suspension

of C. parvum; six days later all six rabbits received intravenous injections of 50 mg NBA. The rabbits were bled as described above and received a second intravenous injection of 50 mg NBA 30 days after the primary injection and bled thereafter for another 30 days.

Primary and secondary responses

The control and C. parvum injected groups were identical. Two rabbits from each group did not produce any detectable anti-BSA antibody after primary or secondary stimulation with NBA. One rabbit from each group produced low levels of anti-BSA antibody in the primary and secondary response but the levels of antibody were too low to determine ABC values.

Conclusion

C. parvum does not facilitate the termination of neonatally induced unresponsiveness to BSA in three month old rabbits.

Experiment 6

The Effects of Corynebacterium parvum Strain 10387 upon the Peripheral White Blood Cell Count, the Phagocytic Index of the Reticuloendothelial System and upon Lymphoreticular Tissue in Rabbits.

The objectives of the sixth series of experiments were to ascertain the physiological and histological effects of C. parvum in the rabbit; it was hoped that such a study would provide some information concerning the mechanisms of the adjuvant activity of C. parvum. The experiments were performed simultaneously with Experiment 3. It was decided to test the effects of C. parvum upon 1) the peripheral white blood cell count, 2) the proliferation of splenic and hepatic tissue, 3) the phagocytic activity of the reticuloendothelial system and 4) the histological changes in the liver, spleen and lung.

Peripheral white blood cell count

The effect of C. parvum upon the peripheral white blood cell counts was studied using the thirty-six rabbits of Experiment 3; Table 3.7 shows the treatments of the various rabbits. Quantitative and differential white blood cell counts were made from blood collected just prior to the injection of C. parvum and/or CBA on day -6, day 0 and day 6 of the primary response. The results of this experiment are given in Table 3.10 which shows the average total white

Table 3.10: Peripheral White Blood Cell Count

	Day 6					Day 0					Day 6				
Group	Total	S.L.	L.L.	Mono.	Poly.	Total	S.L.	L.L.	Mono.	Poly.	Total	S.L.	L.L.	Mono.	Poly.
E 1 E12	8,100	2,100	3,400	1,000	1,600	7.500	1,500	3,600	1,000	1,400	7,500	1,400	4,200	700	1,200
E13 E27	8,200	2,000	3,800	900	1,500	8,700	1,200	5,300	1,100	1,100	9,500	1,600	5,700	900	1,300
E25 E36	8,500	2,400	3,200	900	2,000	7,800	1,000	4,400	1,100	1,300	8,000	1,000	4,500	700	1,800

The data are expressed as number of cells/cubic millimeter of blood. Total, indicates the aggregate white blood cell; S.L. the small lymphocyte; L.L. the large lymphocyte; Mono., the monocyte; and Poly., the polymorphonuclear leucocyte.

blood cell counts and average individual counts for the small lymphocyte, large lymphocyte, monocyte and polymorphonuclear leucocyte; the results are expressed as the number of white blood cells per cubic millimeter of blood. No significant difference between the three groups were noted. In all three groups there was a small decrease in the number of small lymphocytes and a small increase in the number of large lymphocytes on day 0 and day 6. The levels of monocytes and polymorphonuclear leucocytes remained constant.

Spleen and liver weight

The object of this experiment was to determine whether or not C. parvum would cause hepatosplenomegaly. Four New Zealand White rabbits were injected intravenously with 15 mg of a heat-killed suspension of C. parvum; four control rabbits were not injected. Six days later the eight rabbits were killed (after the determination of the phagocytic index) and the weights of the spleen and liver of each rabbit were determined. Table 3.11 shows the design of the experiment and gives the results of the spleen and liver weights. The average body weight of the four C. parvum treated and control rabbits were similar as was the average liver weight. The average spleen weight of the C. parvum treated rabbits was greater than the average spleen weight of the control rabbits. When the average liver and spleen weights were normalized with respect to body weight, it appeared that the C. parvum caused a 7% increase in the liver weight and a 79% increase in the

Table 3.11

Liver and Spleen Weights of *C. parvum* Treated and Control Rabbits

#	Sex	Pretreatment Day - 6	All Weights in Grams Body Wt.	Liver Wt.	Spleen Wt.	L.W./B.W.	S.W./B.W. X 10^3
E40	F	15 mg *C. parvum* I.V.	2,411	92.0	1.6826	.03816	.6979
E41	M		2,965	97.8	1.8832	.03298	.6351
E44	M		2,477	90.8	1.7477	.03666	.7056
E45	M		2,180	81.8	1.7182	.03752	.7882
		average	2,508	90.6	1.7579	.03612	.7009
E42	F	None	2,432	86.4	0.6900	.03553	.2837
E43	M		2,977	92.1	0.5572	.03094	.1872
E46	M		2,895	102.0	1.4223	.03523	.4913
E47	M		2,318	77.5	1.4890	.03343	.6424
		average	2,656	89.5	1.0396	.03370	.3914

All weights are expressed in grams.

average spleen weight.

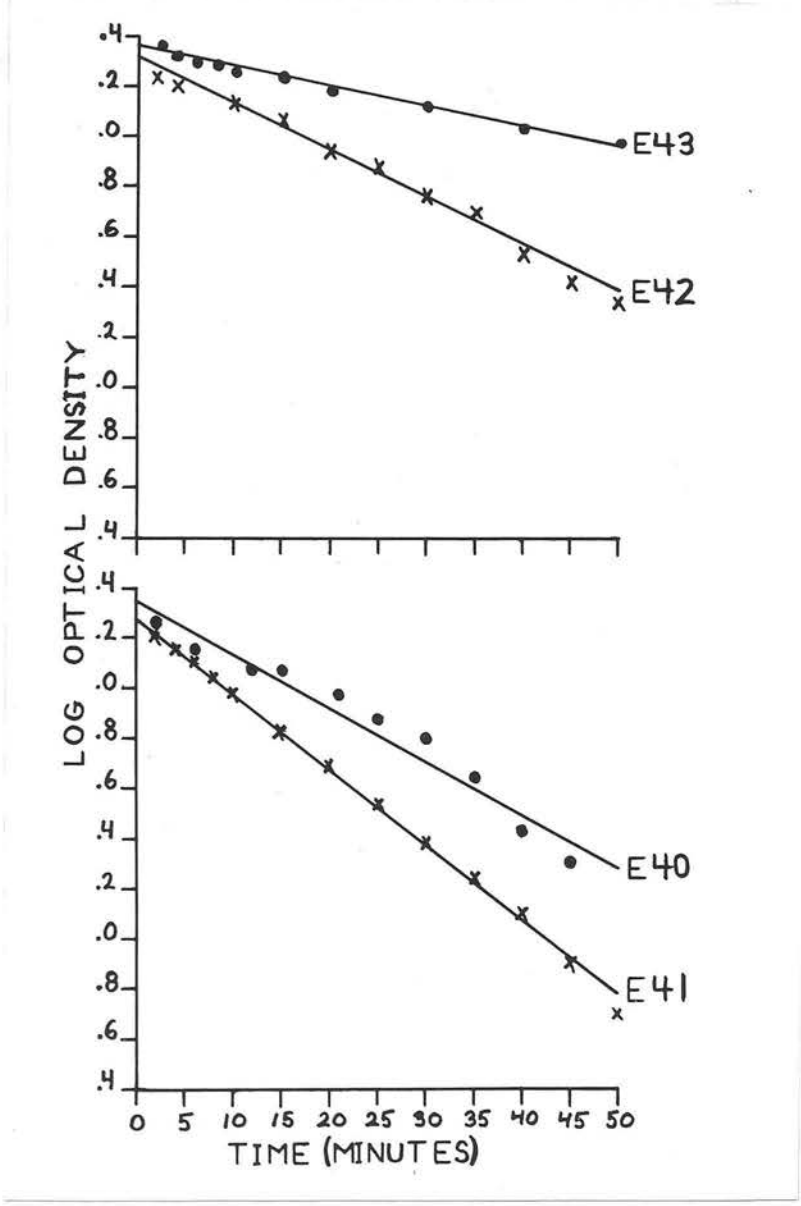
Phagocytic index

The phagocytic indices K and α, determined from the clearance of colloidal carbon from the blood, was studied in C. parvum treated and control rabbits. Two C. parvum treated rabbits, E40 and E41, and two control rabbits, E42 and E43, were used (see Table 3.11). The results of the carbon clearance experiments are shown in Figure 3.11. It was found that the K phagocytic indices of the C. parvum treated rabbits, E40 and E41, were 0.0152 and 0.0304 respectively; the K phagocytic indices of the control rabbits, E42 and E43, were 0.0152 and 0.008 respectively. The corrected phagocytic indices, α, were 6.37 and 9.26 for rabbits E40 and E41 and were 6.92 and 6.42 for rabbits E42 and E43.

Histology of the liver, spleen and lung

Portions of the livers, spleens and lungs of rabbits E40 - E47 were fixed in 10% formalin after the determination of the phagocytic indices and organ weights. Tissue sections were made and stained with Haematoxylin and Eosin. The stained sections from the C. parvum treated rabbits (E40, E41, E44, E45) were compared with the stained sections from the control rabbits (E42, E43, E46, E47). Rabbits E40 - E43 had been previously injected with colloidal carbon which facilitated the characterization of the Kupffer cells in the liver. Table 3.11 shows the treatments of the various rabbits.

Figure 3.11



Rate of carbon clearance from the blood in C. parvum treated (E40) and E51) and control (E42 and E43) rabbits.

Liver histology: The livers from C. parvum treated rabbits contained multiple, small granulomata. They were difficult to observe with the naked eye but were readily seen under the low power objective of the microscope. The granulomata were situated in an irregular manner throughout the lobule although foci, which were mainly lymphocytic in composition, were especially frequent in the portal tracts. A typical granuloma is shown in Figure 3.12 and consists of large numbers of histiocytes with abundant eosinophilic cytoplasm and oval or indented nuclei. Multinulceated giant cells were commonly found containing 8 - 16 nuclei scattered throughout the cytoplasm; a few giant cells contained refractile material of unknown composition. Small numbers of mature lymphocytes and plasma cells were also present but they were greatly outnumbered by the larger histiocytes.

Extensive foci of lymphocytes were also found and these have been called granulomata for convenience, although the relative absence of histiocytes cast some doubt on this terminology. These lymphocytic foci were found mainly in and around the portal tracts where they measured up to 100 microns in diameter. The cells had scanty cytoplasm and large round nuclei of the lymphocyte type. They also extended into the sinusoids where they gave the appearance of budding from the sinusoidal wall (see Fig. 3.13). It might be tentatively suggested that these cells were mediators of an immune response to C. parvum; the higher oxygen tension in the portal tracts might possibly account for their selective

localization in that area. No necrosis of liver was observed in any of the sections.

After the intravenous injection of colloidal carbon, the livers of the C. parvum treated rabbits were strikingly different from the control rabbits. The Kupffer cells of the rabbits which had received C. parvum appeared to be more numerous and larger than in the control rabbits (see Fig. 3.14). No mitotic figures were seen and it cannot be stated whether this appearance is due to an increase in size of the Kupffer cells or an increase in their number.

Spleen histology: The splenic histology was more difficult to interpret and sometimes the spleens from C. parvum treated rabbits contained less carbon than the control rabbits. This was probably due to the increased activity of the liver reticuloendothelial cells after C. parvum stimulation. No granulomata were found in the spleen and it could not be determined whether there were any clear alterations in cell type. The only consistent finding was the increase in the bulk of the white pulp as shown by the larger size of malpighian bodies in the C. parvum treated rabbits (see Fig. 3.15).

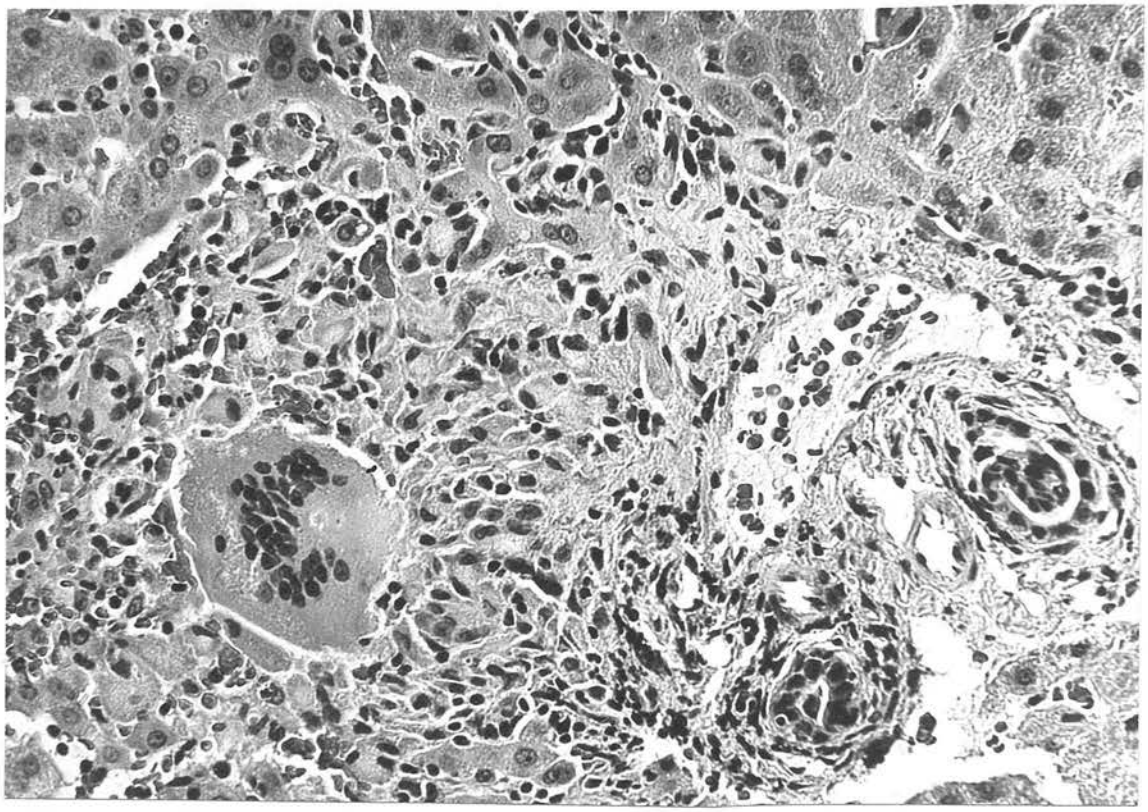
Lung histology: The lungs of C. parvum treated rabbits contained granulomata composed of histiocytes and mononuclear cells with variable numbers of neutrophil polymorphonuclear leucocytes (see Fig. 3.16). Although never being numerous, granulomata could be readily distinguished from the foci of lymphoid tissue which are normally present beside the bronchi.

These granulomata might have been due to infection in the rabbits, but since they were not found in any of the control rabbits, it seems reasonable to ascribe their presence to the lodging of C. parvum particles in the lung after intravenous injection.

Conclusion

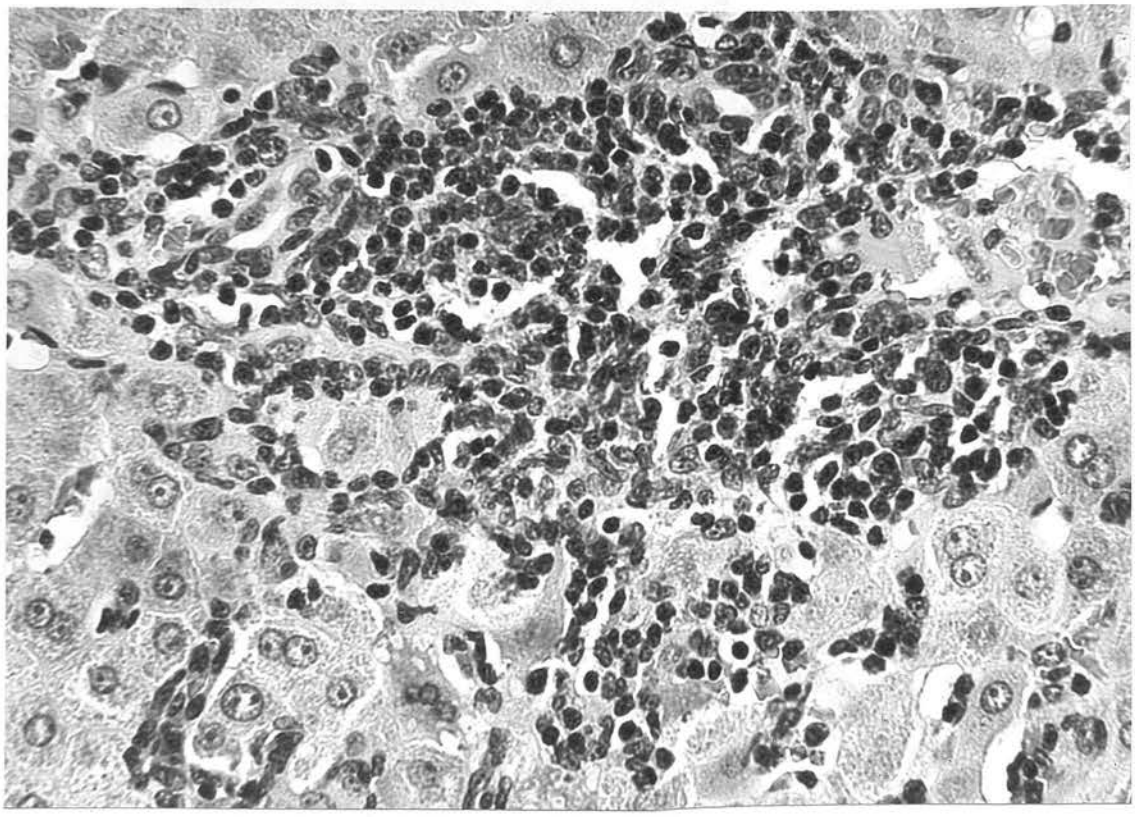
Intravenous injection of C. parvum does not alter the number or distribution of peripheral white blood cells 6 and 12 days later. C. parvum does not cause hepatomegaly but does appear to cause splenomegaly in some rabbits. No conclusions can be drawn concerning the phagocytic index, although it appears that C. parvum does not have a profound effect upon the phagocytic index. Histologically C. parvum has been shown to cause the formation of numerous granulomata in the liver composed of histiocytes, giant cells, lymphocytes and plasma cells. Numerous foci of lymphocytes are also seen mainly around the portal tracts. The Kupffer cells appeared to be increased in number and/or increased in size. C. parvum also caused granulomata formation in the lung and an increase in bulk of the white pulp of the spleen.

Figure 3.12



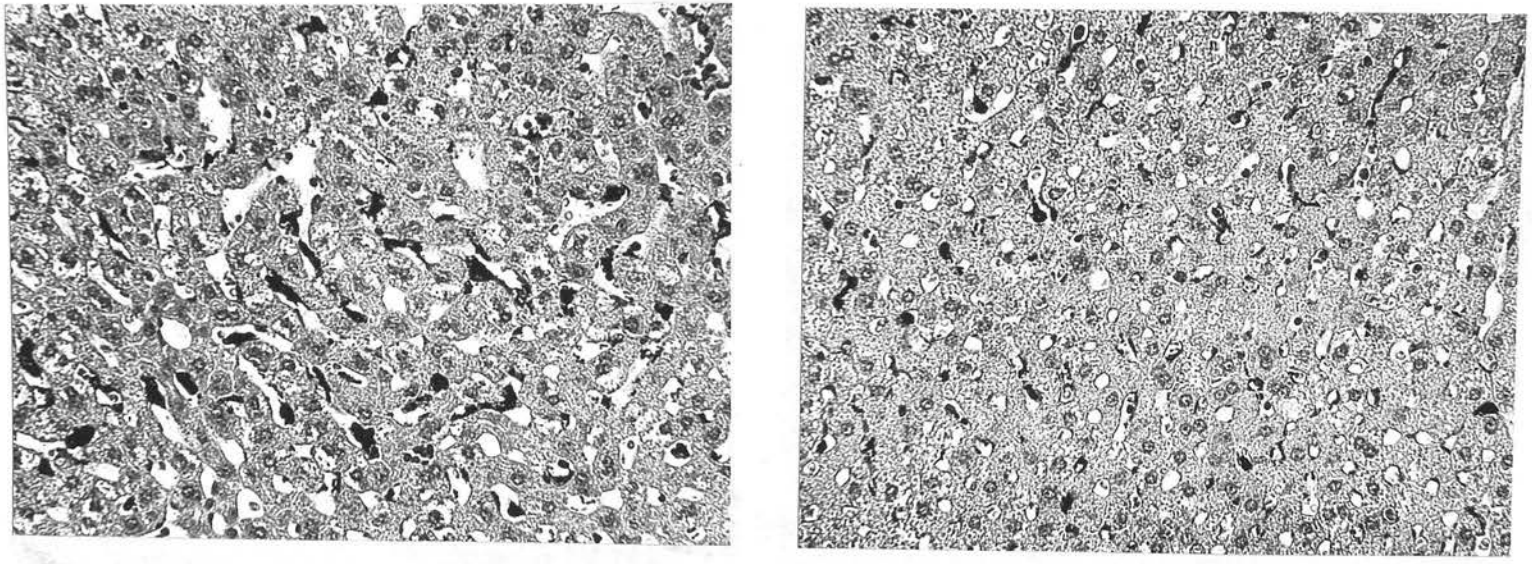
C. parvum granuloma in liver: This shows a multinucleate giant cell of the foreign body type. There are numerous histiocytes with round or oval nuclei and abundant eosinophilic cytoplasm. Small numbers of lymphocytes are also present. Haematoxylin and Eosin X 350.

Figure 3.13



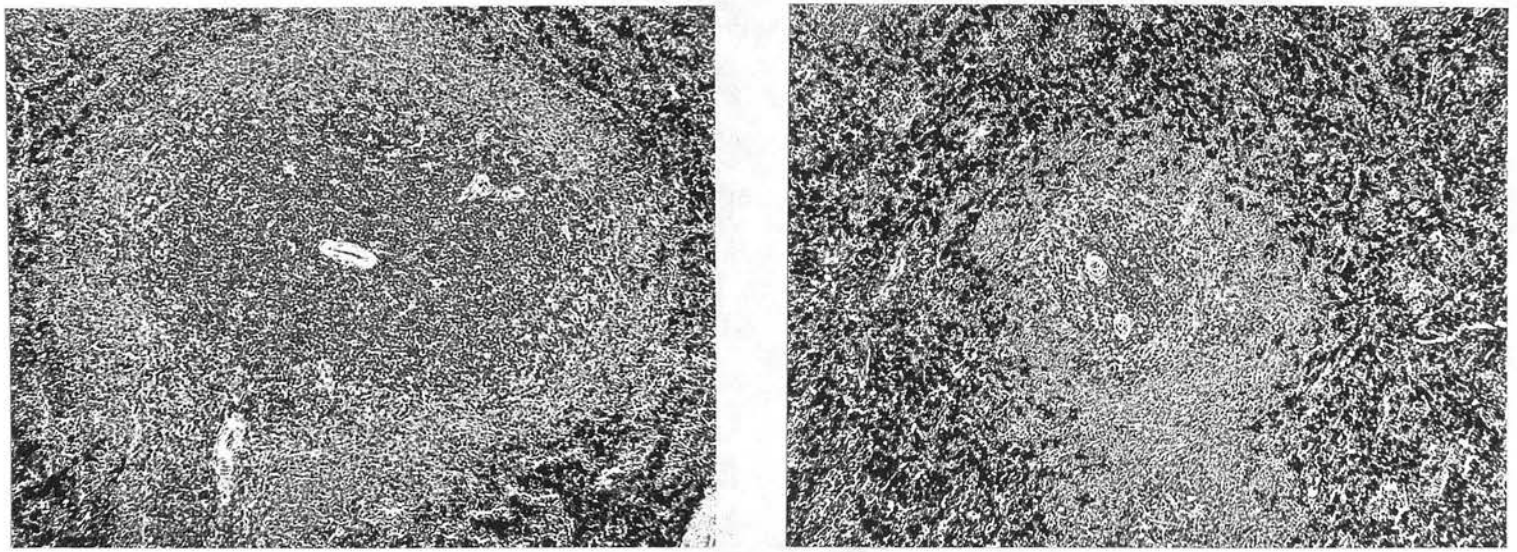
C. parvum granuloma in liver: The response consists chiefly of lymphocytes which are arranged as a solid, central mass with satellite foci extending into the hepatic sinusoids. Haematoxylin and Eosin X 600.

Figure 3.14



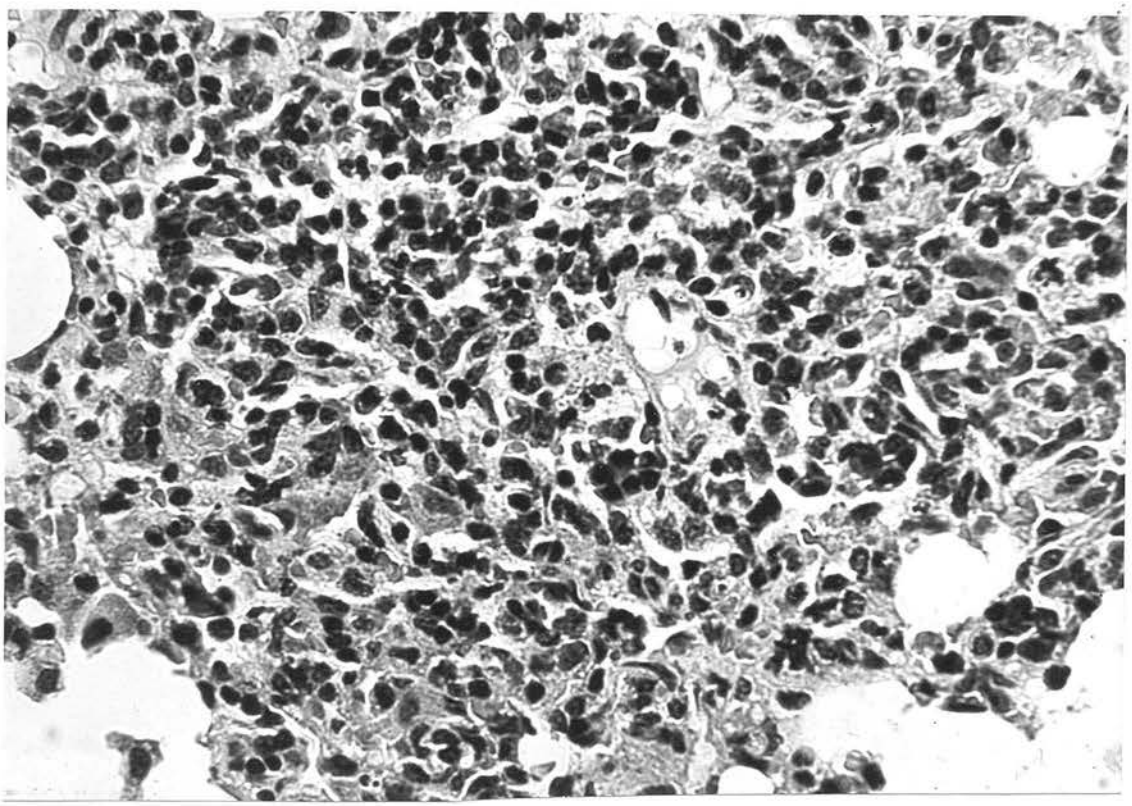
Kupffer cells in the C. parvum treated and control liver: The Kupffer cells in the C. parvum treated (left photomicrograph) appear larger and more conspicuous than in the control liver (right photomicrograph). Haematoxylin and Eosin X 225.

Figure 3.15



Malpighian bodies of the C. parvum and normal spleen: The photomicrograph on the left shows a large malpighian body in a C. parvum treated spleen; the photomicrograph on the right shows a normal malpighian body from a control spleen. Haematoxylin and Eosin X 100.

Figure 3.16



C. parvum granuloma in lung: The granuloma consists mainly of histiocytes and lymphocytes with smaller number of neutrophil polymorphonuclear leucocytes and plasma cells. Haematoxylin and Eosin X 600.

DISCUSSION

Discussion of Experimental Results

The effect of the physical state of BSA upon the quantitative and qualitative immune responses to BSA in the adult rabbit

The physical state of BSA has been shown to have profound effects upon the immune response in rabbits. Centrifuged bovine albumin (CBA) induced a hyporesponsive or unresponsive state to BSA in the majority of adult rabbits. Ten mg CBA appeared to be more efficient in inducing unresponsiveness than 50 mg CBA when the CBA was injected intravenously; 50 mg CBA injected intraperitoneally was also effective in inducing an unresponsive state to BSA. The results indicated that smaller amounts of CBA facilitated the induction of unresponsiveness supporting observations by other workers. Mitchison (1964) found a two dose range of partial tolerance to BSA in adult mice, the lower dose of 10 ug BSA inducing partial tolerance more rapidly than the high dose range of greater than 10 mg BSA. Recently Kraft, Samuelson and Farr (1967) reported a two dose range of antigen-induced immunosuppression facilitated by acriflavine. They found that 1 mg and 1,000 mg BSA would induce partial tolerance when given shortly after treatment with acriflavine; 10 mg and 100 mg, on the other hand, did not induce a profound degree of suppression. It was also indicated that 1 mg BSA was more effective than 1,000 mg BSA in inducing partial tolerance. Nossal, Ada and Austin (1965) found that injections of 1 ug of Salmonella adelaide flagellin into neonatal rats induced a

more lasting state of tolerance than 100 ug flagellin. Rittenberg and Nelson (1963) reported that tolerance to BSA could be induced in rabbits after 400 r whole body X-irradiation only if 10 mg BSA was injected 24 hours later; injection of 100 mg BSA led only to a delayed immune response.

There are some reports which give conflicting evidence for low dose tolerance. Mitchison (1964) found that greater than 10 ug BSA was needed to induce unresponsiveness in 600 r X-irradiated mice. Dixon and Maurer (1955) found that whole body X-irradiation facilitated the induction of tolerance to BSA and human plasma in the adult rabbit using massive doses of the respective antigens. Linscott and Weigle (1964) reported a wide range of tolerogenic doses of BSA in X-irradiated, adult mice; the larger doses of BSA induced a more profound degree of tolerance. Schwartz and Dameshek (1963) found that high doses of antigen were necessary when 6-mercaptopurine was used to facilitate the induction of unresponsiveness to BSA, 66 mg or greater being the necessary amount.

These conflicting reports are difficult to resolve because the induction of tolerance is undoubtedly dependent upon many factors, the species of animal, the antigen being used and the manner of tolerance induction, to mention only three. Secondly, many workers although speaking of tolerance are actually reporting suppressed immunological states. It would appear, however, that a two dose level for the induction of unresponsiveness does exist (Mitchison, 1964) but whether

the mechanisms involved are the same or similar is only a matter of speculation at this time.

In the present study only aggregate-free BSA was able to induce an unresponsive state. These results support other reports where unaggregated antigens had to be employed in order to induce unresponsiveness to relatively low doses of antigen. Dresser (1962a) first reported the induction of tolerance using an aggregate-free antigen. He found that the injection of as little as 50 ug centrifuged BGG would induce tolerance in adult mice; on the other hand 150 mg native BGG was required to produce a similar unresponsive state in adult mice (Dresser, 1962). Battisto and Miller (1962) reported the induction of tolerance to aggregate-free BGG in the guinea pig. Claman (1963) confirmed Dresser's earlier work and demonstrated that from 0.19 mg to 19.0 mg centrifuged BGG induced similar states of tolerance in the mouse.

Dresser and Gowland (1964) met with little success in the induction of tolerance in the adult rabbit to centrifuged horse gamma globulin although 50 mg of centrifuged hare gamma globulin did induce an unresponsive state. Frei, Benacerraf and Thorbecke (1965) also met with limited success using *in vivo* filtered BSA as the tolerance inducing agent, only three of nine rabbits being rendered unresponsive; however, a decreased amount of antibody was noted in the rabbits. Biro and García (1965) were able to induce unresponsiveness in the adult rabbit to HGG after the injection of 5 mg centrifuged HGG; heat-aggregated centrifuged HGG was a powerful antigen

and this demonstrated that centrifugation was not removing potentially antigenic molecules of HGG but only antigen of a different physical state. The work of Biro and García (1965) together with the present findings are the only two reports where a profound degree of tolerance has been induced in the adult rabbit with relatively small amounts of antigen. A possible explanation for this could be the genetic variations amongst rabbits which control reactivity to various antigens (Sobey, Margarth and Reisner, 1967).

The present work has also extended the previously mentioned reports by studying not only the quantity of antibody synthesized but also the quality of the antibody, i.e., the change in relative binding affinity. Only the data from the primary responses will be discussed because the relative binding affinities of the secondary response sera were out of the range of sensitivity of the Farr technique; in order to assess the effect of dilution percentage of secondary sera, antigen concentrations of at least 0.002 ug N and 0.0002 ug N BSA* would have been necessary. In practice it was impossible to use this low range of antigen due to the low radioactivity at this concentration. If the specific activity of the BSA* was increased, radiation damage of the BSA molecule would be likely to have occurred.

The data show that alum-precipitated centrifuged bovine albumin (ACBA) is a powerful antigenic stimulus in the rabbit. This demonstrates, as did Biro and García (1965), that the centrifugation procedure did not sediment chemically different

antigen molecules and that the antigenicity of BSA is dependent upon the physical state of the BSA molecule. The immune response to ACBA differed in two ways from the immune response to CBA: 1) ACBA injected rabbits produced higher and more sustained levels of anti-BSA antibody during the primary and secondary responses; 2) the relative binding affinity of the primary response antibodies from the ACBA injected rabbits generally increased at a more rapid rate and reached higher levels than any antibody produced in the CBA injected groups. These findings indicate that not only can CBA induce unresponsiveness but if antibody is produced the selective mechanisms operating to induce high affinity antibody are also impeded. These results conflict to some degree with the work of Grey (1964). He found that the decrease in the dissociation rate of rabbit anti-BSA antibody was not dependent upon the antigen dose, adjuvants and/or the number of injections of antigen; however, the sera tested were taken relatively later during the immune response and CBA was not employed in this study. Farr (1958) has questioned whether the effect of dilution percentage is influenced by high affinity antibody being selectively removed from the circulation during the primary response. The present report argues against this explanation for two reasons: 1) the effect of dilution percentage was not directly dependent upon the quantity of antibody produced and 2) the effect of dilution percentage values increased similarly reaching the same level whether 10 mg or 50 mg CBA was injected into C. parvum treated rabbits. The

work of Cartsen and Eisen (1955) and Velick, Parker and Eisen (1960) also indicated that the association constants of anti-DNP antibodies were dependent upon the amount of antigen and immunization procedure used, a finding which was later confirmed by Eisen and Siskind (1964). Steiner and Eisen (1966) have recently demonstrated the increase in antibody affinity in the absence of free antigen using *in vitro* techniques. It is also interesting that in the present study native bovine albumin (NBA) induced an immune response intermediary between CBA and ACBA with respect to both quantity and quality of antibody. Although, as previously stated, the secondary response data are difficult to interpret, the increase in the relative binding affinities of the secondary response antibodies in the CBA group are of lower magnitude than the antibodies produced in the NBA or ACBA groups.

The effect of *Corynebacterium parvum* upon the quantitative and qualitative immune responses to BSA in the adult rabbit.

The use of *C. parvum* as a "non-specific" stimulator of antibody production was prompted mainly by the work of Claman (1963). He demonstrated that the injection of 100 ug of *Salmonella typhosa* endotoxin two hours after a normally tolerogenic dose of 1.9 mg centrifuged BGG blocked the induction of tolerance and resulted in a normal immune response. Dresser (1962) had also demonstrated that the induction of high dose tolerance to BGG could be prevented by the injection of BCG at the same time as the injection of the

soluble BGG. Claman and Bronsky (1965) have demonstrated that actinomycin D was able to block the induction of tolerance in adult mice to centrifuged BGG if the actinomycin D was injected for 2 days prior to the injection of centrifuged BGG; 6-mercaptopurine, 5-fluorouracil, nitrogen mustard and cyclophosphamide did not block the induction of tolerance however. C. parvum was chosen as the adjuvant in the present study for two reasons: 1) it was found that C. parvum did not possess detectable toxicity in the newborn rabbit and 2) this organism had been shown to induce massive lymphoreticular cell hyperplasia and was an adjuvant. Therefore any adjuvant activity found could be correlated directly to the lymphoreticular cell proliferation and not to the toxic side effects of endotoxin, actinomycin D or BGG that may result in the release of tissue breakdown products which possess adjuvant properties (Braun, 1965).

The intravenous injection of a heat-killed suspension of C. parvum 6 days prior to the intravenous or intraperitoneal injection of CBA blocked the induction of unresponsiveness and induced a hyperreactive state to BSA characterized by high levels of anti-BSA antibody and a rapid increase in the relative binding affinity of the antibody produced. C. parvum also enhanced the antibody response to NBA when injected 6 days prior to the antigen. Siskind and Howard (1966) have demonstrated that C. parvum when injected shortly before the injection of SII pneumococcal polysaccharide augmented the production of immunity in mice to subsequent challenge with

live pneumococcal organisms as measured by survival rate. Recently Biozzi, Stiffel, Mouton, Liacopoulos-Briot, Decreusefond and Bouthillier (1966) have shown that C. parvum not only caused an increase in the number of immunologically active spleen cells against sheep erythrocytes but also indicated that the amount of antibody synthesized by each cell was increased. The latter finding could also be explained by C. parvum inducing the formation of high affinity antibodies, as indicated from the present data, which might well result in elevated agglutination titres.

It has been shown that C. parvum when incorporated into incomplete Freund's adjuvant induced delayed hypersensitivity in the guinea pig and increased the humoral antibody response to picrylated proteins (Neveu, Branellac and Biozzi, 1964). The induction of delayed hypersensitivity could also be postulated by the production of high affinity antibodies (Karush and Eisen, 1962). Another species of Corynebacterium, C. rubrum, has been shown to possess equal adjuvant properties as Mycobacterium tuberculosis in the production of allergic encephalomyelitis in the guinea pig (Shaw, Alford, Fahlberg and Kies, 1964). Recently Woodruff and Boak (1966) reported that the intravenous injection of C. parvum either 2 days prior or 8 - 12 days after the injection of isogenic, mammary carcinoma cells, resulted in the delayed appearance of the tumour as compared to control mice; they suggested that the inhibition of tumour growth could have been due to an increased immunity to the injected tumour cells. The

injection of C. parvum has also been shown to increase the survival rate of (C57BL/6 x 63H) F_1 hybrid mice from wasting disease after the injection of C57BL/6 spleen cells (Biozzi, Howard Mouton, and Stiffel, 1965); although the increased survival was originally thought to be due to the enhanced resistance to infection, recent evidence suggests that the mechanism of C. parvum protection to the graft-versus-host reaction may not be of an immunological nature (Howard, personal communication).

In the present studies C. parvum did not enhance the primary antibody response to ACBA which by itself is a powerful immunogen. Similarly Howard and Inchley (personal communication) could not demonstrate any adjuvant activity of C. parvum in mice when T_4 coliphage was used as the antigen. These results indicate that C. parvum does not make the cellular mechanisms of antibody formation more efficient if a powerful antigen is employed. On the other hand, C. parvum enables relatively weak antigens to elicit a vigorous immune response. A possible explanation of this phenomenon is that C. parvum decreases the degree of tolerance induction to weak antigens thereby appearing to augment the production of humoral antibody. Mitchison (1964) has similarly implied this view that an immune response is the net result of some cells becoming tolerant and other cells becoming sensitized to an antigen simultaneously after the injection of antigen. The amount of antibody produced would therefore depend upon the ratio of sensitized cells to tolerant cells. The theoretical

mechanisms of antibody formation and the induction of acquired immunological unresponsiveness will be discussed later.

Experiment 2 varied somewhat from Experiment 3 primarily due to the injection of antigen via the intraperitoneal route. The lack of adjuvant effect when C. parvum was injected at the same time as CBA compared with when the organism was given -6 days prior to CBA is inconsistent with Experiment 3 where C. parvum did have an adjuvant effect when injected simultaneously with CBA. Siskind and Howard (1966) also found that augmentation of immunity to SII pneumococcal polysaccharide occurred only when C. parvum was injected 7 days prior to the antigen and not when given simultaneously. There are three explanations for this discrepancy between Experiments 2 and 3: 1) the intraperitoneal route may be more effective in inducing immunological unresponsiveness, 2) in vivo filtering of CBA by the peritoneal macrophages may render the antigen more tolerogenic (Frei, Benacerraf and Thorbecke, 1965) and 3) the simultaneous intravenous injections of C. parvum and CBA in Experiment 3 might have resulted in the absorption of CBA on to the bacterial particles rendering the antigen particulate. As the relative amounts of rabbit serum proteins are in extreme excess to the 10 mg CBA injected, the third possibility seems very remote and therefore factors 1 and/or 2 were most likely to be responsible for the inconsistencies.

One other divergence observed in Experiment 2 was the

striking difference between the secondary response to CBA and ACBA. Whereas particulate ACBA was shown to be more effective than CBA in inducing primary antibody stimulation the opposite was the case in the secondary response. A possible explanation for this is that, compared with the injection of ACBA by the intravenous route, the secondary intraperitoneal injection of ACBA was phagocytized by the peritoneal macrophages and thus did not come in contact with sensitized lymphoid cells in the spleen and lymph nodes bringing about their proliferation. On the other hand, a large proportion of CBA presumably passed without phagocytosis to the lymphatic system. This view is consistent with the observations of Uhr and Bauman (1961) that passively administered antibody given during secondary antigenic stimulation suppressed the immune response by encouraging phagocytosis of the antigen.

The effect of Corynebacterium parvum strain 10387 upon lymphoreticular tissue in the adult rabbit

The injection of 15 mg of a heat-killed suspension of C. parvum strain 10387 did not produce the profound degree of lymphoreticular cell proliferation as had been anticipated. The peripheral white blood cell levels remained relatively constant 6 and 12 days after the injection of C. parvum with respect to the total white cell counts and the percentages of small lymphocytes, large lymphocytes, monocytes and polymorphonuclear leucocytes. No hepatomegaly was observed and

minimal splenomegaly occurred. The injection of C. parvum strain 10387 into mice has also been shown not to cause hepatosplenomegaly whereas the injection of C. parvum strain 936B induced a two-fold increase in the liver weight and a six-fold increase in the spleen weight (Howard and Inchley, personal communication). The corrected phagocytic indices also were not significantly increased. Histologically C. parvum induced granulomata formation in the liver and lung mainly composed of histiocytes with smaller numbers of lymphocytes and plasma cells. Numerous lymphocytic foci were observed in the portal tracts and the Kupffer cells of the liver were either increased in number and/or in size. Few histologic changes were noted in the spleen except for a possible increase in the bulk of the white pulp. No necrosis was noted in any of the sections observed.

The above description is not the classical picture which has been reported for strain 936B by the French research group. C. parvum strain 936B has been shown to induce considerably enhanced phagocytic activity of the reticuloendothelial system and massive hyperplasia of lymphoid tissue (Halpern, Prévot, Biozzi, Stiffel, Mouton, Morard, Bouthillier and Decreusefond, 1964). The injection of only 255 ug of C. parvum strain 936B into mice caused in 8 days an increase in the phagocytic index of 770% and induced a two-fold weight gain in the liver. Histology of the liver contained an increased number of Kupffer cells and numerous nodules (granulomata) in the portal spaces caused by infiltration of histiocytic elements. In

the spleen there was a three-fold weight gain, extensive hyperplasia of the red and white pulp, increased numbers of histiocytes and lymphocytes in the lymphoid follicles and an increased number of giant cells. Although there are many histologic similarities between the two strains of C. parvum, strain 936B has by far the more powerful stimulatory action upon the lymphoreticular tissue; the differences in the lymphoreticular proliferative activity between different strains of C. parvum is not an uncommon finding (Biozzi, personal communication).

In view of the reduced activity of C. parvum strain 10387, it is difficult to postulate possible mechanism(s) of adjuvant action. One possibility which has been ruled out is that C. parvum might conceivably share antigenic determinants with the BSA molecule. Rabbits which received four monthly injections of 15 mg of a heat-killed suspension of C. parvum did not produce detectable anti-BSA antibody as determined by the Farr technique using 0.02 ug N BSA*. When the rabbits were later injected with 50 mg NBA they elicited a normal primary antibody response indicating that priming had not occurred. As no necrosis was observed in any of the histologic sections, the possibility of tissue breakdown products acting as an adjuvant can also be ruled out. There are a number of other possibilities but which are at present all speculative. The numerous granulomata in the liver and lung might provide additional centres of antibody formation, thereby making the induction of tolerance more difficult;

however, splenectomy suppresses the immune response in mice which have liver granulomata produced by BGG (Howard, personal communication). C. parvum might through lymphoid hyperplasia induce high levels of "natural antibody" or opsonins which might facilitate the early mechanisms of antibody production. The physiologic function of the lymphoreticular cells could also be altered by C. parvum, particularly the macrophages. The adjuvant effects of many substances still remain an obscure area of immunology. Whatever are the specific mehcnaism(s), the present study indicates that C. parvum either accelerates the "recognition phase" and/or "triggering mechanisms" of antibody formation or renders the lymphoid cells less susceptible to tolerance induction.

The second effect of C. parvum on the immune response, outside of blocking the induction of unresponsiveness and augmenting antibody production, was to increase the rate of evolution of the relative binding affinities of the antibodies produced. It is again difficult to critically evaluate these findings at this time. The increase in the affinity of antibody after immunization can best be described by a selective mechanism of the proliferation of those sensitized lymphocytes capable of synthesizing high affinity antibody. C. parvum could potentiate such a mechanism by 1) blocking the induction of tolerance in the potentially high affinity antibody clones or daughter cells and/or 2) non-specifically C. parvum could increase the lymphoid cell population from which selection of high affinity antibody would be favoured especially if the

quantity of antigen available to cause specific lymphoid proliferation was limited.

The effect of Corynebacterium parvum on the induction and abrogation of neonatally induced unresponsiveness to BSA in the rabbit

The intraperitoneal injection of 2 mg of a heat-killed suspension of C. parvum into newborn rabbits did not impede the induction of unresponsiveness induced by the intraperitoneal injection of 100 mg NBA 6 days later. Therefore C. parvum did not bring about an increase in the rate of maturation in the immune system of the neonatal rabbit. The injection of bacterial endotoxins simultaneously with BSA into neonatal rabbits or the injection of BSA into BGG infected neonatal rabbits did not interfere with the induction of tolerance (see Smith, 1961). Whether these findings may be related to the blockage of tolerance induction by C. parvum in the adult rabbit is questionable. The neonatal rabbit is immunologically immature; peripheralization of lymphatic tissue is not complete, the permeability of cellular membranes is greater than in adult rabbits and the blood thymus barrier is not fully established (Good and Papermaster, 1964; Elves, 1966). Secondly, 1,000 times more antigen was injected into the neonatal rabbit thus producing a "high dose" unresponsive state in contrast to the "low dose" unresponsive state induced in the adult rabbit.

The intravenous injection of 15 mg of a heat-killed

suspension of C. parvum followed 6 days later with 50 mg NBA into three month old rabbits, which were previously rendered unresponsive to BSA in the neonatal period, did not facilitate the abrogation of the tolerant state. These results were not surprising as previous reports, which attempted to abrogate tolerance by injection BSA-adjuvant mixtures, met with minimal success (Smith, 1961). Freund's complete adjuvant facilitated the termination of BSA tolerance in adult rabbits only when azo-BSA was used; if BSA was injected simultaneously with the azo-BSA the unresponsive state was not terminated (Weigle, 1964a).

If cell turnover is an important factor in the natural breakdown of tolerance (Mitchison, 1965) one might expect C. parvum and other bacteria to facilitate the abrogation of the unresponsive state due to the proliferation of lymphoid tissue. The cell turnover and termination of tolerance appear to be thymic dependent (Claman and Talmage, 1963; Taylor, 1964) and therefore it would seem that lymphopoiesis is necessary in order to terminate tolerance. Whether C. parvum could directly effect the thymic control over lymphopoiesis is doubtful due to the blood thymus barrier in the adult rabbit; C. parvum appears only to cause proliferation of pre-existing lymphoid elements and therefore would not increase the turnover of lymphoid cells derived from precursor elements. In any case the relatively inferior lymphoreticular cell proliferative effects of C. parvum strain 10387 might possibly explain the failure to abrogate tolerance in the

present study. Siskind and Howard (1966) also were unable to terminate a pre-existing state of paralysis to SII pneumococcal polysaccharide in mice with C. parvum.

Conclusions

The degree of aggregation of bovine serum albumin (BSA) has been shown to influence not only the quantity of antibody produced but also the magnitude and rate of increase of the relative binding affinity of the antibodies as characterized by the Farr technique. The injection of low doses of centrifuged, aggregate-free BSA into adult rabbits induced either a hyporesponsive or unresponsive state to BSA. Alum-precipitated, centrifuged BSA on the other hand produced a hyperreactive state and native BSA, which is partially aggregated, led to an intermediary immune response.

Intravenous injections of a heat-killed suspension of Corynebacterium parvum strain 10387 into adult rabbits, either six days prior to or simultaneously with an intravenous injection of centrifuged BSA, initiated "non-specific" factors in the immune system enabling the aggregate-free BSA, which normally induced an unresponsive or hyporesponsive state, to induce a hyperreactive response; if the centrifuged BSA was injected intraperitoneally, C. parvum was only effective when injected six days prior to the antigen. Neonatal rabbits, injected intraperitoneally with C. parvum less than twelve hours after birth and with 100 mg BSA six days later, became unresponsive to BSA to the same degree as control rabbits

which did not receive an initial injection of C. parvum. It has also been shown that C. parvum was unable to facilitate the termination of unresponsiveness to BSA (induced in the neonatal rabbit) in three month old rabbits.

The injection of C. parvum strain 10387 into adult rabbits did not initiate the characteristic, massive lymphoreticular cell proliferation or increase in the phagocytic index as reported for other strains of this bacterium. C. parvum also did not significzntly alter the quantitative or differential white blood cell counts six and twelve days after the intravenous injection of the organism. Histologically the livers of C. parvum treated rabbits contained multiple granulomata composed mainly of histiocytes with smaller numbers of lymphocytes and plasma cells; the number and/or size of Kupffers cells in the liver was also increased. The lungs contained granulomata and the spleens appeared to have an expanded white pulp. No necrosis was observed in any of the tissue. From the above it would therefore appear, that the adjuvant effect of C. parvum must either be due to an increase in "natural opsonins" and/or an alteration in the physiology of the cells involved in the early stages of the immune response.

Theoretical Discussion of the Mechanisms of Antibody Formation and the Induction of Acquired Immunological Unresponsiveness

Mechanisms of antibody formation

At this time in the adolescence of immunology, discussion of the mechanisms of antibody formation and the mechanisms of the induction of acquired immunological unresponsiveness can only be highly speculative. At present immunologists are still divided into at least two schools of thought concerning the mechanism of antibody formation: 1) the Lamarkian school instituted by Haurowitz, Mudd and Alexander and 2) the Darwinian school instituted by Ehrlich, Jerne and Burnet. The intricacies of the arguments of the two schools of thought are highly sophisticated and are strictly the province of the molecular biologist and the animal geneticist. The following discussion must of necessity be brief and of a fairly superficial nature.

The instructive theories of antibody formation are at first the most attractive because they permit flexibility of the immune system enabling the production of antibodies to a wide variety of naturally occurring or synthesized antigens. The experimental evidence for these theories is poor, being derived to a great degree from analogy with other biological systems, the induction of enzyme synthesis being only one example. The template theory has been virtually excluded because of the recent evidence indicating that the primary

structure of the antibody molecule largely determines the secondary and tertiary structure of the globulin molecule. Therefore one must look to the DNA dependence of antibody synthesis which suggests a clonal theory (Darwinian) or antibody production.

The clonal theory of antibody production was not established from direct studies of antibody synthesis but in order to explain the induction of immunological tolerance. It postulated genetically pre-determined clones of cells for every antigen; if therefore clones of pre-determined cells were eliminated during the embryonic period by a "self-marker" recognition mechanism the induction of tolerance can easily be explained. The main argument against this theory is the multitude of antigenic determinants some of which yet may not even have been synthesized. The answer to this objection is the panacea of the clonalists, i.e., the existence of somatic mutation occurring in the adult animal; thus the clonal-selection theory was established (Burnet, 1959) eliminating the necessity of pre-determined clones. However, problems immediately arise for this interpretation. Humphrey (1964) found that rabbits rendered tolerant to BSA during the neo-natal period did not recover reactivity to BSA for many months, even after the disappearance of detectable antigen from the circulation. This would indicate that the mutation rate in the rabbit must be very low (Humphrey, 1965) and thus it is difficult to envisage how somatic mutation could account for the thousands of antigenic determinants, especially in a

non-rapidly dividing population of cells.

The increase in the relative binding affinity of antibody after the injection of antigen may also provide indirect evidence concerning the mechanisms of antibody formation. The association constants of antibodies to a single antigenic determinant have been shown to vary over at least a 10,000 fold range during the course of several weeks after antigenic stimulation. This rapid increase in affinity, irrespective of the instructive or clonal theories, can be explained by the selective proliferation of those cells producing high affinity antibody. It is known, however, that high affinity antibody is structurally different from low affinity antibody in that high affinity antibody incorporates a larger portion of the light chain in the antibody combining site (Hong and Nisonoff, 1966). Because antibody, directed towards the same antigenic determinant, possesses differing affinities and differs with respect to primary structure, the clonal theory becomes less tenable. The existence of thousands of "sub-clones" within the main clone must be postulated; as a conservative estimate this might well raise the number of pre-existing clones to greater than 10^{10}. The other explanation of the clonalists would be somatic mutation; if this were true the mutation rate would have to be exceedingly high in order to account for such a rapid increase in the association constants. The increase in affinity of antibody, by the clonal selection theory, would not only depend upon the mutation rate but also on the number of lymphocytes within a given animal. If one

therefore assumed that the number of lymphocytes in the elephant exceeded the number of lymphocytes in the mouse by a factor of 10^6, the clonal selection theory would predict that the elephant would be more efficient in producing high affinity antibody if the mutation rates in the two animals were similar. If, on the other hand, the increase in antibody affinity was similar for the elephant and mouse, one would have to postulate that the mutation rate in the mouse was 10^6 times greater than the mutation rate in the elephant. Both predictions in the author's opinion do not appear physically plausible. Although it is unlikely that the immune response of the elephant and mouse will be assessed, the reasoning nevertheless casts doubt upon somatic mutation solely being responsible for the production of new clones of lymphocytes. In any case, many immunologists and geneticists today do not feel that any feasible mutation rate can account for the diversity of antibody formation (R. Clayton, personal communication).

One possible explanation for the diversity of antibody specificity and the increase of antibody affinity would be a "clonal-random variation" mechanism taking the following form. The author is prepared to accept that "specific" antibody clones must exist initially in order to account for the induction of tolerance. However, it is postulated from the above discussion that the various clones are likely to be multipotent for related groups of antigenic determinants. The injection of antigen would proliferate the multipotent

clone and the sensitized cells would become more specialized producing higher and higher affinity antibody through cellular variations at the transcriptional and/or translational levels of protein synthesis; a similar view has been presented by Braun (1965a).

Support for the multipotent clone may be indirectly obtained from the recent study on antigen competition by Kim, Bradley and Watson (1966). They demonstrated that the neonatal piglet, which is immunologically competent at birth, is highly susceptible to antigen competition even through dietary antigens. Austin and Nossal (1966) found that the induction of tolerance in the neonatal rat to one flagellar antigen inhibited the response to a later challenge with another chemically unrelated flagellar antigen. Direct evidence for the multipotent clone was reported by Feldman and Mekori (1966) who demonstrated that "cloned-cell" populations of lymphocytes were pluripotent to a greater degree than the present author has proposed.

The mechanisms of antibody formation are and are likely for some time to be an enigma to the immunologist. The explanation for this state of affairs is the lack of an operational approach to study such mechanisms; the current theories of antibody formation for the most part have arisen through analogy rather than from observation. The clonal-selection theory provides an apparently simple explanation but there are many arguments against it especially concerning the necessary high mutation rate. The "multipotent-clone" theory

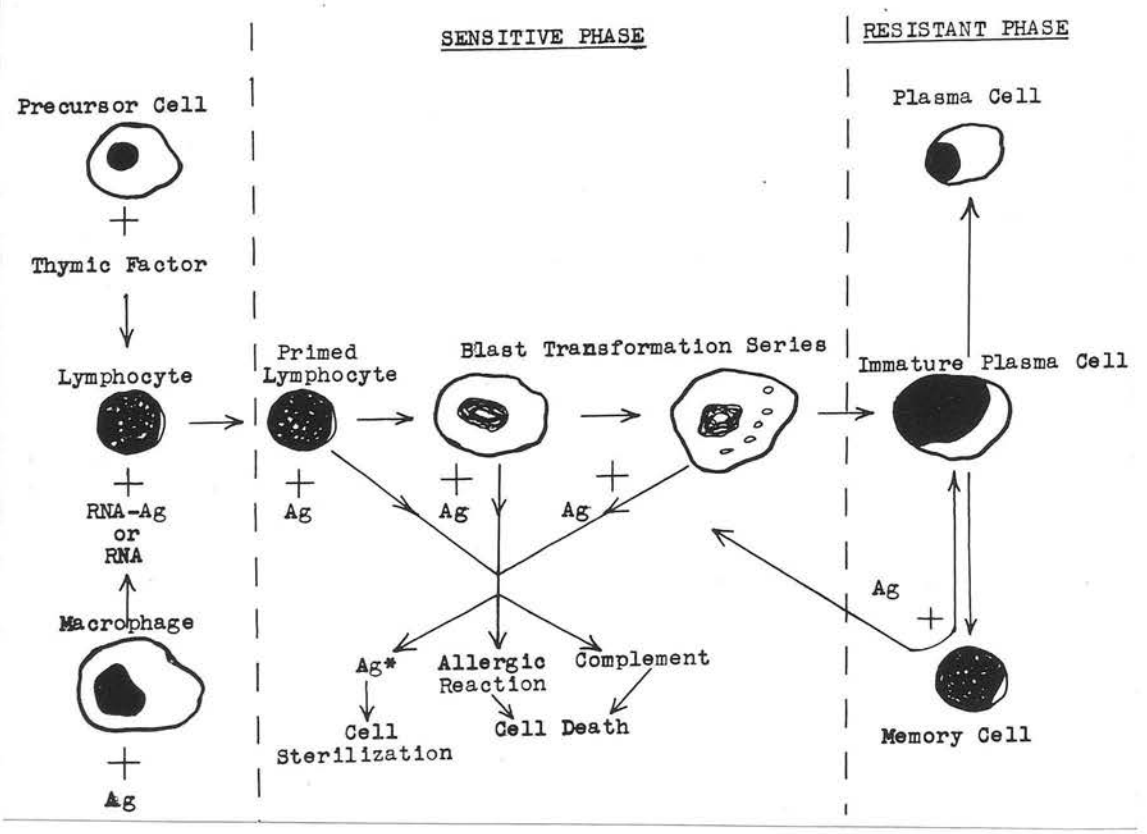
with cellular variations occurring at the transcriptional and/or translational level of protein synthesis would provide an explanation for the extremely efficient mechanism for antibody formation.

Mechanisms of tolerance induction

The induction of unresponsiveness in the author's opinion can only be adequately explained by "clonal-deletion". The question which immediately follows is what are the specific mechanisms of clonal-deletion? Table 4.1 gives three possible mechanisms for the induction of tolerance. It is postulated that a sensitive phase and a relatively resistant phase of tolerance induction exists. The present study and other reports have shown that the low dose tolerance to aggregate-free serum proteins is relatively easy to induce. It is assumed that although antibody is produced during the sensitive tolerance induction period, the transformation processes are not completed and thus plasma cells and memory cells are not produced. If, on the other hand, plasma cells and memory cells are produced, one must use more drastic measures to induce tolerance (Dorner and Uhr, 1964; Dresser, 1965); therefore this is termed the resistant phase.

Three possible mechanisms for the induction of tolerance through clonal deletion are given in Table 4.1; the three mechanisms apply only to the sensitive phase and no account will be given of the induction of tolerance in the resistant phase although the mechanisms may possibly be of a similar

Figure 4.1



Theoretical representation of three possible mechanisms for the induction of immunological unresponsiveness.

Ag^{*} indicates an antigen fragment.

nature. The antigen is processed by the macrophage and either RNA (Fishman, van Rood and Adler, 1965) or RNA-Ag (Askonas and Rhodes, 1965) is transferred to the uncommitted lymphocyte which then becomes a "primed" lymphocyte initiating the production of small amounts of antibody which tends to adhere to the cell surface due to continual, slow release. Antigen then interacts with antibody on the cell surface of the primed lymphocyte inducing the blast transformation series resulting in the production of mature plasma cells and memory cells. This would be the mechanism of antibody formation provided that three possible events do not occur during the blast transformation steps.

The primed lymphocytes and/or blast cells could react with a modified form of antigen (perhaps a univalent fragment) which would inhibit further transformation or proliferation thereby eliminating the clones after the sensitized cells undergo normal death; this mechanism has been termed "cell sterilization". The second possible mechanism of clonal deletion could occur through an "allergic reaction" (possibly of the delayed type) resulting in the death of the cell; Rich and Lewis (1932) reported what appears to be such a phenomenon. They demonstrated that spleen cells from guinea pigs previously sensitized with Mycobacterium tuberculosis, when cultured in vitro with old tuberculin, would undergo rapid death whereas normal guinea pig spleen cells grew normally in the presence of old tuberculin even if anti-M. tuberculosis antiserum was added to the culture.

The third and preferred mechanism involves the interaction of antigen and antibody at the cell surface of a primed lymphocyte and/or blast cells and the fixation of complement which would result in the lysis of the cell. There is indirect evidence for this hypothesis. Makela and Mitchison (1965) demonstrated, by the adoptive transfer of sensitized spleens or lymph node cells to unsensitized, X-irradiated, isogenic mice, that tolerance could be induced in the recipient mice by high doses of antigen; however, if the sensitized cells were incubated with antigen *in vitro* for 6 - 24 hours, washed and injected into the recipient mice, enhanced antibody formation occurred rather than the induction of tolerance. This work indicates the importance of the *in vivo* environment upon the induction of tolerance, which could well be the presence of complement. Work is presently being carried out, using the experimental design of Makela and Mitchison (1965), in order to determine the effect of complement upon the adoptive transfer of immunity, by incubating the sensitized spleen cells with antigen and complement prior to injection into the X-irradiated recipients.

The postulated "sensitive" and "resistant" phases of tolerance induction could be different with respect to the apparent difference in the secretion of antibody by the lymphocyte and the plasma cell. It appears that the small lymphocyte and perhaps the large lymphocyte produce antibody which adheres to the surface of the cell presumably due to a constant, slow secretory mechanism. Sell (1967) has recently

shown that specific sheep antibody to the gamma chains of rabbit IgG and the mu chains of rabbit IgM induced the blast transformation of 100% of rabbit thoracic duct lymphocytes; it was also demonstrated that specific sheep anti-Fc fragment of rabbit IgG was also able to induce blast transformation (Sell, 1967a). The immuno-cyto-adherence technique (Biozzi, Stiffel, Mouton, Liscopoulos-Briot, Decreusefond and Bouthillier, 1966) has also demonstrated antibody on the surface of small and large lymphocytes (Biozzi, personal communication). From the work of Sell (1967a) it is likely that antigen interacting with antibody on the surface of the lymphocyte would bind complement through the exposed Fc portion of the antibody molecule resulting in the lysis of the cell. This theory also becomes more attractive in that the small lymphocyte is capable of synthesizing IgM antibody; it has been demonstrated that only one molecule of IgM antibody is needed to cause lysis of a red cell (Humphrey and Dourmashkin, 1965) and therefore the lymphocyte would be particularly susceptible to cell lysis.

The plasma cell and perhaps certain immature plasma cells and blast cells appear to be able to store synthesized antibody in the ergastoplasm channels, secreting the antibody only under certain conditions (see Holub, 1967). This mechanism of antibody release therefore might reduce the susceptibility of these cells to lysis; IgG antibody is predominantly synthesized by the plasma cell family which also would reduce the chances of lysis of the cells compared to the IgM

synthesizing small lymphocytes. It is difficult to postulate if the memory cells, which appear to be small lymphocytes, are very susceptible to lysis; this would depend upon the degree of dedifferentiation which would occur in the transition to a mature plasma cell.

Nossal and Mitchell (1966) have proposed a similar hypothesis of the mechanism of tolerance induction caused by the lysis of an antibody synthesizing lymphocyte interacting with antigen and complement. They further have postulated that if antigen is bound to the dendritic macrophage, clonal deletion would not occur. The mechanisms of action of C. parvum in inhibiting the induction of unresponsiveness can only be a matter of speculation at the present time. It could function by increasing the localization of antigen capture in the dendritic webs of specialized macrophages by non-specifically augmenting the levels of "natural antibodies". C. parvum could also accelerate the early mechanisms of antibody formation thereby resulting in the earlier development of the cells in the resistant phase of tolerance induction. Dresser (1962a) has shown that the induction of tolerance takes at least 3 - 4 days which lends support to the tolerance-inhibitory action of C. parvum accelerating antibody formation.

Studies on the induction of acquired immunological tolerance and of changes in the relative binding affinities of antibody, in the author's opinion, are two of the most powerful tools which may help to elucidate the mechanisms of antibody formation. The present work has studied some factors which

influence the induction of tolerance and the increase in the relative binding affinity and the preliminary findings presented are encouraging; they have also suggested many other areas of related study. Above all, the most important lesson derived from this dissertation by the author has been a realization of the need for an integrated research programme encompassing two or more experimental approaches, directed towards a common goal. The simultaneous study of the effects of the physical state of an antigen and/or changes in the lymphoreticular tissue upon the induction of unresponsiveness and upon the quantity and quality of antibody produced was designed to make a start on such an integrated programme in order to elucidate the mechanisms of antibody formation.

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